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Effect of Feeding Moderate Levels of Commercially Agenized Flour to Dogs.*

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During the past 18 months many questions have been raised concerning the role of nitrogen trichloride-treated flour (agenized) in the etiology of human epilepsy. Shortly after Erickson *et al.*[1] suggested that wheat gluten might be important in the causation of human epilepsy, a report by Mellanby[2] showed that epileptiform convulsions in dogs could be consistently induced when the animals were fed rations which contained high amounts of flour treated with nitrogen trichloride. These observations were later confirmed by Silver *et al.*[3] and Newell *et al.*[4] and the studies were extended to other animals. In all these experiments the rations fed to dogs contained large amounts of flour (70-84%). These flours were treated with nitrogen trichloride in amounts which ranged up to 15 times more than the treatment normally given flour consumed by human beings.

The use of agene as a maturing agent has increased steadily during the past 25 years. In 1945, 85% of the flour used in this country was treated with nitrogen trichloride. Because of the wide use of wheat products, it was decided to place a series of dogs on a diet which contained an amount of flour approximately equal to that used in the average American diet. This flour was treated with nitrogen trichloride at commercial levels commonly used by the milling industry.†

The following ration was used: flour 30, sucrose 49, casein 10, corn oil 5, salts IV 4, whole liver substance 2. Oral supplements of 0.75 mg thiamine, 0.75 mg riboflavin, 0.5 mg pyridoxine, 0.25 mg pantothenic acid, 1 mg nicotinic acid and 70 mg choline per kilo of body weight and 6 drops of halibut liver oil were administered twice weekly. Three dogs, 19, 23 and 30), were placed on ration F-89 which contained flour treated with 1.2 g nitrogen trichloride and 7 g benzoyl peroxide‡ per cwt. of flour. Two other dogs, (241 and

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[1] Erickson, T. C., Gilson, W. E., Elvehjem, C. A., and Newell, G. W., *Proc. Assn. Research in Nervous and Mental Dis.*, 1946, **26**, 164.

[2] Mellanby, E., *Brit. Med. J.*, 1946, **2**, 885.

[3] Silver, M. L., Johnson, R. E., Kark, R. M., Klein, J. R., Monahan, E. P., and Zevin, S. S., *J. Am. Med. Assn.*, 1947, **135**, 757.

[4] Newell, G. W., Erickson, T. C., Gilson, W. E., Gershoff, S. N., and Elvehjem, C. A., *J. Am. Med. Assn.*, 1947, **135**, 760.

† We are indebted to Dr. H. K. Parker of Wallace and Tiernan Company for the flours used in these experiments.

244), were started on ration F-89A which contained flour treated with 2.25 g of nitrogen trichloride and 7 g of benzoyl peroxide[‡] per cwt. of flour.

Dogs 19 and 23 have been on ration F-89 for 63 weeks (14½ months) and dog 30 has been fed the same ration for 55 weeks (12½ months). Dogs 241 and 244 have been fed ration F-89A which contains 30% flour treated with the higher level of nitrogen trichloride, for 52 weeks (12 months). All of these animals have eaten well, gained weight and remained healthy. At no time has there ever been any indication of running fits or epileptiform convulsions.

Before this experiment was begun characteristic electroencephalographic brain wave patterns (E.E.G.s) of dogs 19, 23 and 30 were obtained.[§] Four tantalum electrodes were implanted into the skull above the dura of each animal in the manner described by Newell *et al.*[5] Normal E.E.G. records were obtained for each of these animals. Six to 8 months after these experiments started tantalum electrodes were implanted in all 5 dogs. A week later several E.E.G. records were taken and all the animals showed normal electroencephalograms. In Fig. 1 short sections of the record from one electrode lead of dog 30, characteristic of the entire record of this dog and of the other dogs, are presented to show the similarity of the E.E.G.s before the feeding tests began and during the period of testing. Previous experiments, in which dogs have received flour or wheat gluten treated with high levels of nitrogen trichloride, have consistently showed abnormal changes in the E.E.G. records days before running fits or epileptiform convulsions developed.[5] If any abnormalities had developed in the brain metabolism or physiology of the dogs fed rations containing 30% of flour treated with agene at commercial levels, significant changes in the E.E.G. records should have been readily observed.

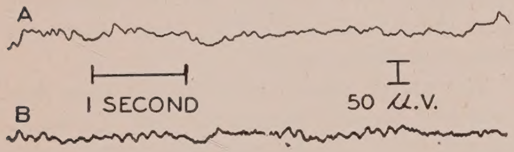

FIG. 1.

A—Control electroencephalogram of dog 30 before experiment.

B—Electroencephalogram of dog 30 after 6½ months on a ration which contained 30% of commercially agenized flour.

It is interesting to note here that the flour used in these experiments when fed to dogs at 84% of the ration, regularly caused running fits and convulsions in 11-62 days.[4] This difference in flour intake suggests that there is a tolerance level in the dog below which the toxic factor produced by nitrogen trichloride treatment is metabolized or excreted but above which the toxic material accumulates in the animal producing the characteristic convulsive condition. When higher levels of nitrogen trichloride are used to treat flour, smaller amounts of flour induce the convulsions. Radomski *et al.*[6] have recently shown that dogs fed 0.1 g of nitrogen trichloride treated wheat gluten (6 g NCl_3/kg gluten) per kilo of body weight per day did not develop running fits. These authors translate their results to mean that a dog could consume 25% of his ration in the form of commercially treated flour (1.5 g NCl_3/cwt.) without developing fits.

Summary. These results demonstrate that dogs can be fed a ration containing moderate levels (30%) of commercially agenized flour for long periods of time (12-14½ months) without developing running fits or electroencephalographic abnormalities.

[‡] Previous tests have shown benzoyl peroxide bleached flour to be ineffective in producing fits.[4]

[§] We wish to express our thanks to Dr. Henry M. Suckle for his assistance in some of the experiments.

[5] Newell, G. W., Erickson, T. C., Gilson, W. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 115.

[6] Radomski, J. L., Woodard, G., and Johnston, C. D., *Fed. Proc., Abstracts*, 1948, **7**, 250.

16596

Lipotropic Effect of Liver Extract on Dietary Hepatic Injury in Rats.*

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Many different supplements have been studied for their effect in preventing liver injury produced by dietary means. The possible effect of liver extract, however, has received little attention. Rhoads and Miller[1] observed that liver extract only partially restored to normal the abnormal liver function produced by a black-tongue diet in dogs. György and Goldblatt[2] studied the effectiveness of a liver concentrate in preventing dietary cirrhosis in rats and concluded that the "liver extract was completely ineffective in preventing hepatic injury". Gillman and Gillman[3] reported that liver extract had some lipotropic effect on the fatty livers of infants with pellagra. In view of the lack of experimental data on the effect of liver extract on experimental dietary liver disease, it was felt that further studies should be made.

Methods. Male rats of the Sprague Dawley strain, weighing between 150 and 170 g were used. All animals received food *ad libitum.* Five animals of each group were housed in individual cages, and their daily food intake was measured. The diet selected was one recently used by Himsworth and Glynn[4] which is high in fat but not low in protein and which produced fatty changes followed by fibrosis. The diet consisted of 16% casein, 51% lard, 30% cornstarch, and 3% salt mixture. Vitamins A and D were supplied by adding 5 drops of oleum percomorphum§ per kilo of diet to the melted lard as the diet was prepared. Each rat received a daily oral supply of the B vitamins as follows: thiamin, 20 μg; riboflavin, 25 μg; pyridoxine, 20 μg; calcium pantothenate, 100 μg. One group of 13 rats received the basic diet alone. The second group of 10 rats received a supplement of 1 cc of Lilly's Crude Liver Extract‡ (1 unit per cc) 3 times a week subcutaneously. A third group of 10 animals received 40 mg of choline chloride daily given subcutaneously in a 4% solution.

After 12 to 13 weeks of the study, a liver biopsy was performed on the 5 animals in each group in the individual cages. The biopsy was carried out by laparotomy under ether anesthesia, and tissue was taken from the edge of the left lobe. At 22 weeks a second biopsy was performed on the same animals in a similar manner. At 29 weeks all surviving animals were killed, and sections were taken for histological examination from the left lobe and the caudate lobe of the liver. The system of grading histological changes is listed with Table I. All liver sections were stained with hematoxylin and eosin. In addition, for half of the animals in each group, frozen sections were made and stained with Sudan III. The remaining liver was analyzed for total lipids by a modification of the

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† Post-War Fellow, Rockefeller Foundation, 1947-1948.

‡ Present address: Department of Physiology and Pharmacology, Wayne University College of Medicine, Detroit, Mich.

[1] Rhoads, C. P., and Miller, D. K., *J. Exp. Med.*, 1938, **67**, 463.

[2] György, P., and Goldblatt, H., *J. Exp. Med.*, 1942, **75**, 355.

[3] Gillman, T., and Gillman, J., *Arch. Int. Med.*, 1945, **76**, 63.

[4] Himsworth, H. P., and Glynn, L. E., *Clin. Science*, 1944, **5**, 93.

§ The authors wish to thank Dr. K. K. Chen of Eli Lilly and Company for the Liver Extract and Dr. C. E. Bills of Mead Johnson and Company for the Oleum Percomorphum. Each cubic centimeter of liver extract contained: Thiamin, 2.0-4.5 μg: Riboflavin, 45.0-60.0 μg; Pantothenic acid, 45.0-60.0 μg; Nicotinic acid, 100.0-140.0 μg; Folic acid, 2.0-3.8 μg; Methionine, 3.0 μg; and Choline, 1.0-1.6 μg. Total solids, 16-18%.

TABLE I.
Histological Changes in Liver at Biopsy and Necropsy.

Basic diet					Liver extract					Choline				
		Fat					Fat					Fat		
Rat No.	Wt change	L.L.	C.L.	Fibrosis	Rat No.	Wt change	L.L.	C.L.	Fibrosis	Rat No.	Wt change	L.L.	C.L.	Fibrosis
Biopsy at 12-13 weeks.														
1	+66	4+		0	14	+ 66	0		0	24	+175	0		0
2	+56	4+		0	15	+ 99	0		0	25	+126	0		0
3	+55	3+		1+	16	+ 90	0		0	26	+110	0		0
4	+74	3+		0	17	+100	0		0	27	+ 71	0		0
5	+32	3+		0	18	+124	0		0	28	+112	0		0
Biopsy at 22 weeks.														
1	+80	4+		1+	14	+ 81	0		0	24	+179	0		0
2	+58	4+		1+	15	+107	0		0	25	+159	0		0
3	+60	4+		2+	16	+106	0		0	26	+116	0		0
4	+67	3+		0	17	+111	0		0	27	+ 71	0		0
5	+23	3+		0	18	+127	0		0	28	+112	0		0
Necropsy at 29 weeks and earlier deaths.														
1	+58	4+	4+	2+	14	+ 48	0	0	0	24	+ 42	0	0	0
2	+28	3+	3+	2+	15	+ 65	0	0	0	25	+ 50	0	0	0
3	—16	4+	4+	2+	16	+ 52	0	0	0	26	+ 52	0	0	0
4	+30	4+	4+	2+	17	+ 50	0	+	0	27	+ 65	0	0	0
5	+ 3	4+	4+	0	18	+ 68	0	+	0	28	+ 35	0	0	0
6	+56	3+	3+	1+	19	+ 57	0	0	0	29	+ 59	0	0	0
7*	—32	4+	4+	2+	20	+ 82	0	+	0	30	+ 88	0	0	0
8*	+22	4+	4+	2+	21	+ 86	0	0	0	31	+108	0	0	0
9*	+ 3	4+	4+	1+	22	+ 98	0	+	0	32*	+ 86	0	0	0
10*	+26	4+	4+	1+	23	+ 28	0	0	0	33*	+ 52	0	+	0
11*	+40	4+	4+	0										
12*	+14	4+	4+	2+										
13*	+10	4+	4+	2+										

*Comments.

No. 7 died at 148 days of pneumonia.
No. 8 ” ” 167 ” from anesthesia.
No. 9 ” ” 67 ”—no infection found.
No. 10 ” ” 70 ”—” ” ”
No. 11 ” ” 72 ”—” ” ”
No. 12 died at 165 days of pericarditis.
No. 13 ” ” 170 ” ” ”
No. 32 ” ” 175 ” ” pneumonia.
No. 33 ” ” 186 ” ” ”

Grading of Fatty Change.

0—None or only a rare large droplet.
+—A few large droplets in each lobule.
++—More than +, but less than half of lobule involved.
+++—Over half of lobule involved but some cells fat free.
++++—Almost no fat free cells.
L.L. = Left lobe of liver; C.L. = Caudate lobe of liver.

Grading of Fibrosis.

0—None.
+—Thin strands of fibrous tissue scattered throughout.
++—Strands of fibrous tissue dividing liver into small sections.

method of Outhouse and Forbes.[5]

Results. The untreated animals fed the high-fat diet showed a marked increase in liver fat histologically, whereas treatment with liver extract or choline prevented the fatty changes in the liver (Table I). As indicated in Table I several animals died prior to the 29th week, generally from liver injury associated with infection.

In the untreated animals the change observed at the first biopsy was a marked infiltration with large fat droplets which almost completely filled the hepatic cells, pushing the nucleus and remaining cytoplasm into a narrow rim. The end result seen in the autopsy sections was a trabeculation of the entire liver with strands of fibrous tissue dividing the liver into sections smaller than the normal lobule. Large, isolated, fat-free hepatic cells with heavily staining cytoplasm and one or two large, dark staining nuclei were occasionally seen enmeshed in the fibrous strands. The progressive nature of the fibrosis in the untreated animals may be observed in the data in Table I. The fibrosis was never present unless there was also a marked fatty change. In following those animals with marked fatty change and fibrosis through biopsies to autopsy it was observed that as the fibrosis increased there was a slight decrease in the amount of fat in the cells.

The animals treated with liver extract or choline did not show any fatty change in the left lobe of the liver at the first or second biopsy or at autopsy. However, in a few of the animals in these groups additional examination of the caudate lobe at necropsy showed a slight (1 +) fatty change (Table I). However, this is a normal variation between lobes of the liver as other animals fed a normal diet of 16% casein, 6% fat, 75% corn starch, and 3% salt mixture showed a similar variation between left and caudate lobes in some cases.

TABLE II.
Total Liver Lipids in % of Wet Weight.

Basic Diet		Liver Extract		Choline	
Rat No.	% Fat	Rat No.	% Fat	Rat No.	% Fat
1	27.3	14	8.5	24	8.9
2	25.8	15	10.4	25	6.2
3	18.2	16	7.5	26	7.6
4	27.1	17	6.4	27	7.4
5	20.1	18	7.1	28	8.3
6	14.3	20	11.9	30	8.3
8	20.4	21	9.2	31	10.2
12	15.1	22	11.2	32	5.4
13	15.9	23	7.4	33	3.9
Mean	20.50		8.84		7.36
S.E.	±1.72		±.65		±.64

The results of the lipid determinations are shown in Table II. The untreated animals showed a definite increase in lipid concentration. Treatment with liver extract or choline prevented the increase in liver lipids, and the resulting concentration of lipids in these animals are in the same range as rats fed a normal low-fat diet (16% casein-6% fat). Lipid determinations were not performed on the animals that died early in the experiment or who showed postmortem changes.

Discussion. Previous investigators have produced similar hepatic lesions of fatty change and fibrosis in rats by dietary means.[4,6] Others have produced a dietary injury consisting of fatty change, fibrosis, and necrosis[2,4,7,8] Necrosis was not observed in any of the animals in the present study. All of the diets that have produced necrosis differ from that used here in several aspects. An important difference that is usually associated with the occurrence of necrosis is the use of diets which are low in protein, generally ranging from 6% to 10% protein. In the present study the diet contained 16% protein.

The supplement of choline prevented both the infiltration of the liver with fat and the fibrosis which followed the fatty change in untreated animals. This lipotropic effect of choline is well known, but a lipotropic effect of liver extract as obtained in the present study, has not been reported previously.

György and Goldblatt[2] studied the effect of daily doses of 0.2 g of a liver concentrate

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[5] Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, 1940, **25**, 1157.

[6] Blumberg, H., and Grady, H. G., *Arch. Path.*, 1942, **34**, 1035.

[7] Handler, P., and Dubin, I. N., *J. Nutrition*, 1946, **31**, 141.

[8] Webster, G. T., *J. Clin. Invest.*, 1942, **21**, 385.

TABLE III.
Average Daily Intakes of Food, Protein, and Calories.

Treatment	Food g/rat	Protein g/rat	Protein G per 100 g rat	Calories per rat	Calories per 100 g rat
Basic diet	7.2	1.15	0.56	46.3	22.4
Liver extr.	8.0	1.28	0.54	51.6	21.6
Choline	9.1	1.45	0.55	58.5	22.1

in preventing the hepatic injury of fatty change, cirrhosis and necrosis produced in rats by a low-protein, high-fat diet. They concluded that the liver extract failed to prevent the induced liver injury. Either the differences in the types of injury produced, or in the liver concentrate used by György and Goldblatt may be responsible for the lack of effect of their concentrate as compared with the beneficial effect of liver extract in the present study. The animals in the present study received on the basis of total solids the equivalent of 0.51 g of powdered liver No. 343 per rat per week as compared to the 1.4 g of concentrate used by György and Goldblatt.

The effect of the liver extract does not appear to be due to an increase in protein intake resulting from stimulation of the appetite by the liver. The protein intake per rat was only slightly higher in the liver-treated group as compared to the untreated group, and the protein intake per 100 g of rat weight was the same in both groups (Table III). A group of animals not included in this report were given the basic diet with an additional 8% casein. This resulted in an average protein intake per rat of 2.12 g per day, and a protein intake of 0.79 g per 100 g of rat weight per day. Even with this protein intake, which is considerably above that of the liver extract and choline treated animals, all of these animals showed a 1+ or 2+ infiltration with fat.

The choline content of the liver extract is not responsible for its effectiveness. The liver extract used contained 1.0-1.6 mg of choline per cubic centimeter, and therefore each rat received only 0.56 mg of choline a day. Other data in the literature show that larger amounts of choline are necessary to prevent dietary lesions in the liver. The lowest effective dose used was that of Daft, Sebrell and Lillie[9] who found that a dietary supplement of 20 mg of choline protected against the lesion produced by a 4% protein-5% fat diet. Himsworth and Glynn[4] found that the fat content of the liver was normal at 34 days, if 4 mg of choline daily was used as a supplement to a diet containing 8% protein and 50% fat; but abnormal amounts of fat were present at 66 days in spite of continued choline administration. Furthermore, other supplements which will be reported in detail later, and which failed to prevent the fatty change, had a higher content of choline than the liver extract. It is possible that the liver extract contains an unknown lipotropic substance.

Summary. 1. Rats fed a 16% casein, 51% fat diet for 29 weeks all developed markedly fatty livers. Nearly all of the animals showed a diffuse, progressive hepatic fibrosis.

2. The fatty change and fibrosis were completely prevented by supplementing the high-fat diet with choline, or with crude liver extract.

3. The lipotropic effect of the liver extract is not due to an increase in protein intake resulting from a stimulation of appetite, and is not due to the small amount of choline present in the liver extract.

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[9] Daft, F. S., Sebrell, W. H., and Lillie, R. D., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 228.

16597

Failure of Splenectomized Rabbits to Respond to Liver Extract and to Pteroylglutamic Acid.*

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Jacobson and Williams[1,2] reported that splenectomized rabbits responded to the administration of several materials with an increase in reticulocytes. Liver extracts effective in the treatment of pernicious anemia, synthetic xanthopterin, leucopterin and the pigment derived from fused uric acid caused reticulocytosis in their animals. Clinically inactive liver extracts as well as a number of unrelated materials failed to evoke a response. Substances inducing reticulocyte increases were effective when given either intramuscularly, intravenously or orally. Based on a large number of control counts it was concluded that any reticulocyte value exceeding 3.5% following administration of a trial material indicated hemopoietic activity of that material. It is to be noted that none of their animals was anemic at any time during the period of observation.

The nature of the materials found effective by these authors stimulated us to test in a similar fashion the newly synthesized pteroylglutamic acid (PGA)[3] which had been found to be effective in the treatment of the macrocytic anemia of sprue[4] and in pernicious anemia.[5]

Procedure. Nine adult white rabbits were used. The animals were fed Purina rabbit chow exclusively throughout the period of experimentation. Eight were males. The spleen was removed from 6 of these animals; the remaining 3 served as normal controls. Of the 6 splenectomized rabbits 4 were treated and 2 were followed as splenectomized controls. There was very little loss of blood from operation. However, to avoid reticulocyte increases owing to blood loss, 3 weeks were allowed to pass following operation before any trial material was given. Preoperative and post-operative red blood cell counts and determinations of hemoglobin indicated no anemia by standards reported for rabbits.[6]

Rabbits No. 1 and 2 (splenectomized) were given 5 mg of PGA intramuscularly on 4 occasions over a period of 8 days. Rabbits No. 3 and 10 (splenectomized) were given 5 units of liver extract (Wilson) intramuscularly on 3 occasions over a period of 7 days. No reticulocyte increases having occurred by 10 days following the last injection of a series the animals were re-treated after a rest period of 10 days. Animals No. 1 and 2 were given 20 mg of PGA intramuscularly on 3 occasions within a 5 day period. Rabbits No. 3 and 10 were given 20 units of liver extract (Wilson) intramuscularly on 3 occasions over a period of 5 days. No reticulocyte responses having followed, liver extract and PGA were then given into the stomach by means of a tube. Animals No. 1 and 3 were given 50 units of liver extract (Wilson) by tube on 3 occasions over a period of 7 days. Animals Nos. 2 and 10 were given 10 mg of PGA by tube on 3 occasions over a 7 day period.

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* This work was supported by a grant from The National Vitamin Foundation.

[1] Jacobson, W., and Williams, S. M., *J. Path. Bact.*, 1945, **57**, 101.

[2] *Ibid.*, 1945, **57**, 423.

[3] Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1945, **102**, 227.

[4] Darby, W. J., and Jones, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 259.

[5] Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D., *J. Lab. and Clin. Med.*, 1945, **30**, 1056.

[6] Wintrobe, M. M., Shumacker, H. B., Jr., and Schmidt, W. J., *Am. J. Physiol.*, 1936, **114**, 502.

With few exceptions daily reticulocyte counts were made on the 4 splenectomized animals throughout the entire periods following the first treatment. These periods covered approximately 7 weeks. Splenectomized and normal controls were followed with practically daily reticulocyte counts over a period of 3 weeks from the beginning of the experiment. The wet technic for counting reticulocytes was employed using brilliant cresyl blue dye. One thousand cells were counted on each determination. The same 3 observers made all counts. The liver extract used was known to be potent in the treatment of pernicious anemia.

Results. No significant increase in reticulocytes was noted in any of the animals which has been splenectomized and adequately treated with either liver extract or PGA. The normal controls showed reticulocyte ranges from .6 to 5.3%; the splenectomized controls showed ranges of 1.3 to 4.8%; the animals treated showed ranges of .7 to 5.2%, the values bearing no relation to material administered. Chart 1 shows the reticulocyte range of a normal control animal. Chart 2 shows the range of a splenectomized control. No charts are shown for rabbits treated since the reticulocyte ranges of these animals differed in no respects from those shown. The reticulocyte ranges given were experienced by practically all of the animals used, regardless of group. No trend in any direction was noted in the case of a single animal.

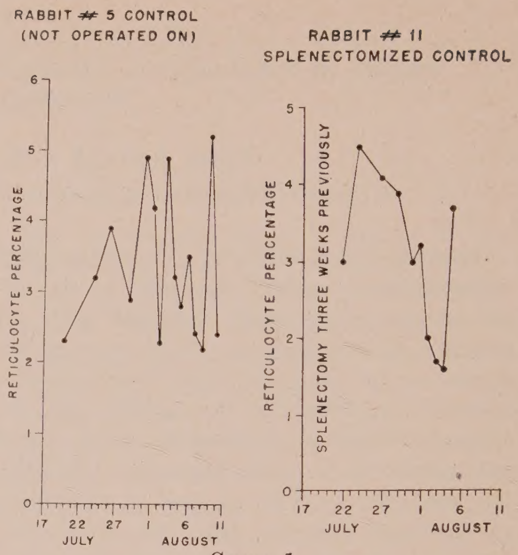

CHART 1.

Conclusions. Splenectomized white rabbits showed no significant change in percentage of reticulocytes following oral and parenteral administration of either pteroylglutamic acid or of liver extract effective in pernicious anemia. Greater variations were found in daily reticulocyte counts, both in controls and in splenectomized animals, than were reported by Jacobson and Williams.

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Observations on Isosensitization to the M Agglutinogen in Man.

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It is generally considered that the agglutinogens M and N can be disregarded when selecting donors for blood transfusion therapy. While this is true for patients requiring only a single transfusion, or multiple transfusions within a short space of time, in cases where several transfusions spaced over a long period of time (months or years) are required, one must take into account the possibility of isosensitization to the M agglutinogen as well as the Rh factor.

Only a relatively small number of cases have been reported of type N individuals with natural anti-M agglutinins in their sera.[1,2] In addition, a few cases have been encoun-

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[1] Wiener, A. S., *Blood Groups and Transfusion*, 3rd ed., p. 219, C. C. Thomas, Springfield, Ill., 1945.

[2] Unger, L. J., Wiener, A. S., and Sonn, E. B., *Am. J. Clin. Path.*, 1946, **16**, 45.

tered with anti-M agglutinins produced apparently as a result of transfusions of type M blood into type N individuals. Wiener and Forer[3] reported a case in which a woman had hemolytic reactions following blood transfusions, and difficulty was encountered in finding a completely compatible blood in cross-matching tests. On testing the blood of the patient, these investigators found her blood to belong to group O, type N, Rh-negative, while her serum contained anti-M and anti-Rh agglutinins. Broman[4] has reported a hemolytic transfusion reaction caused by M sensitization.

In analyzing our cases of Rh sensitization, we found that the spacing of injections was far more important in bringing about isosensitization than the quantity of antigen introduced into the body.[5] In fact, we have applied this principle successfully in devising a simple method of producing anti-Rh serum in normal male type rh donors.[6] While these investigations on the preparation of anti-Rh sera were being carried out, some observations were made on the isosensitization of normal male individuals to the M agglutinogen. The purpose of this paper is to describe these observations.

Case I. This group AB, type rh volunteer was given repeated intravenous injections of 2 cc each of whole Rh-positive blood diluted to 5 cc with sodium citrate solution. The first (priming) injection was given in October 1946. The second injection, or first "stimulating" injection, was given in the middle of February 1947. Ten days later the donor's serum was found to contain univalent Rh_0 antibodies (Rh_0 glutinins) of 50 units titer by the plasma conglutination method, together with weak anti-rh″ agglutinins. Since the titer was not high enough to yield a usable serum, the donor was not bled at that time, and a second stimulating injection was given 2 months later. After this injection the Rh_0 glutinin titer was found to be 85 units by the plasma conglutination method, while the anti-rh″ agglutinin titer rose to 20 units. At this time, we also detected in the volunteer's serum a weak agglutinin reacting with Rh-negative blood but not with the donor's own blood cells. The donor was given a fourth injection of 2 months later, after which the anti-Rh_0 glutinin titer was 512 units by the albumin-plasma method, while the anti-rh″ agglutinin titer proved to be 40 units. Again the presence of a third antibody reacting with type rh blood was noted, and it was decided to investigate the nature of the third antibody.

Titrations showed the third antibody to be an agglutinin of 3 units titer, approximately equally active at body, room and refrigerator temperatures. Tests were set up against a series of 28 blood samples of type rh and type Rh_1, but otherwise unselected, by the agglutination method at room temperature.* Of the 28 bloods tested, only 2 gave negative reactions, besides the volunteer's own blood. One of the negatively reacting blood samples belonged to type $ONRh_1rh$ and the other to type BNrh. Since these 2 blood samples were the only ones in the series besides the volunteer's which belonged to type N, this suggested that we were dealing with an anti-M immune agglutinin. In fact, further tests proved that all type N bloods of types Rh_1 and rh were not agglutinated by the serum, while all other bloods were agglutinated, confirming the diagnosis of anti-M as the specificity of the antibody.

For subsequent stimulating injectings only type N, Rh-positive blood was used, and as shown in Table I, later bleedings obtained from this volunteer contained no demonstrable M antibodies.

Case II. The second volunteer, also group AB and type rh, has not been studied as intensively as the first one. Unlike the first volunteer, this individual produced a potent

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[3] Wiener, A. S., and Forer, S., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 215.

[4] Broman, B., *Acta Paediat.*, 1944, **31**, supp. 2.

[5] Unger, L. J., and Wiener, A. S., *Am. J. Clin. Path.*, 1945, **15**, 280.

[6] Wiener, A. S., and Sonn-Gordon, E. B., *Am. J. Clin. Path.*, 1947, **17**, 67.

* The Rh_0 glutinin in the serum did not clump type Rh_1 bloods under these conditions. Bloods of types Rh_2 and Rh_1Rh_2 could not be used in these tests because of the anti-rh″ agglutinin in the serum.

TABLE I. Results of Immunization Experiment on Two Type N, Rh-Negative Individuals.

		Rh$_0$ antibodies (titer in units†)					
			Univalent antibodies as determined by				
				Conglutination test in			
Case history	Date*	Agglutinins (titration in saline media)	Blocking test	Plasma	Albumin-Plasma mixture	Anti-rh″ agglutinins (titration in saline media)	Anti-M agglutinins (titrations in saline media§)
Volunteer No. 1, A$_1$BNrh	2/26/47	0		50		5	0
Blood used for injection OMRh$_2$Rh$_2$	3/ 5/47	0	4	85		2	0
	4/29/47	0	4	85		20	2
	6/18/47	0	4	104	214	16	3
	6/25/47	0	6		512	40	3
	8/27/47	0	12		500	12	0
	12/ 5/47	0			300	40	0
	1/30/48	0	8			160	0
	2/ 9/48	0			192	64	0
Volunteer No. 2, A$_2$BNrh	8/28/47	640				0	0
Blood used for injection OMRh$_0$	3/ 1/48	70‡			192	0	8

* These tests were usually made 10 days after a stimulating injection.

† The number of units equals the reciprocal of the highest serum dilution giving a one-plus reaction.

‡ Clumping weak throughout. § Tests for anti-M done at refrigerator, room, and body temperatures (no difference in titer).

anti-Rh$_0$ agglutinin of 640 units after the first stimulating injection. Following a second stimulating injection about 6 months later, the Rh$_0$ agglutinin titer was 70 units but there were also Rh$_0$ glutinins of a titer of 192 units by the albumin-plasma method, as well as an additional agglutinin of 8 units titer which reacted with type rh blood cells. Specificity tests proved the third antibody to be an anti-M immune agglutinin.

Comment. The observations just described demonstrate the necessity of administering only type N blood to type N patients requiring a series of transfusions at widely spaced intervals. When injections are spaced at sufficiently wide intervals, the agglutinogen M seems to be a fairly good antigen for individuals of type N. This was well illustrated by a case concerning which we were consulted several years ago, because of difficulty encountered in the cross-matching tests. This we found to be due to an anti-M immune agglutinin which was active in dilutions as high as 1:256 at body temperature. On the other hand, the N agglutinogen appears to be a weak antigen for individuals to type M. Only a single convincing case of isosensitization to the N agglutinogen has been reported[7] in the literature and this involved a patient with an extraordinary capacity to produce antibodies, who in response to a series of transfusions formed in succession antibodies specific for factor hr′, N, and 3 previously undescribed agglutinogens.

With regard to the previous report by Singer[8] claiming isosensitization to the N agglutinogen by transfusion, only a few bloods were tested with the patient's serum and this is not adequate to establish the specificity of an abnormal antibody. Natural anti-N agglutinins were found by the late G. L. Taylor in one case (cited by Callender and Race[7]) and another such example was reported by Allott and Holman.[9] The case reported by

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[7] Callender, A. T., and Race, R. R., *Ann. Eug.*, 1946, **13**, 102.

[8] Singer, E., *Med. J. Aust.*, 1943, **2**, 29.

[9] Allott, E. N., and Holman, C. A., *Lancet*, 1947, **2**, 130.

TABLE II.
Analogies Among the A-B-O, M-N, and Rh-Hr Systems of Blood Factors.*

System*	Major agglutinogens (More antigenic)	Minor agglutinogens (Less antigenic)
A-B-O	A, B, A_1, C†	$O(A_2)$‡
M-N	M	N
Rh-Hr	Rh_0, rh′, rh″	Hr_0, hr′, hr″

* For simplicity the rare agglutinogens A_3, N_2, rh‴, etc., are not included in this table.
† The factor C is shared by bloods containing agglutinogens A_1, B, or both.
‡ The factor $O(A_2)$ is shared by agglutinogens O and A_2, but absent from agglutinogens A_1 and B.

TABLE III.
Allelic Relationships Among the Landsteiner Blood Groups and Subgroups.

	Reaction with serums		Blood groups	
C-O genotypes	Anti-A_1,B (Anti-C)	Anti-A_2.O (Anti-O)	Phenotypes	Genotypes
CC	+	—	A_1, B, A_1B	A_1A_1, BB, and A_1B
CO	+	+	A_1, B, and A_2B	A_1O, A_1A_2, BO, and A_2B
OO	—	+	O, A_2	OO, A_2A_2, and A_2O

DeKromme and Van der Spek[10] also seems to be an example of natural anti-N rather immune anti-N, as claimed by these workers, considering that their abnormal antibody had the properties of a cold agglutinin.

As is well known, in the Rh-Hr system, the Rh and Hr agglutinogens are related to one another serologically and genetically like the M and N agglutinogens.[1] Of the Rh-Hr agglutinogens, the Rh factors are the more potent antigens and thus correspond to the agglutinogens M in the M-N system, while the Hr factors are the less potent antigens and correspond to the agglutinogen N (cf. table II). That similar relationships also exist in the A-B-O groups is demonstrated in Table II and Table III.

Summary. Two cases are described in which type N individuals produced anti-M agglutinins following injections at widely spaced intervals of blood containing the M agglutinogen. On the basis of these and other observations, it is pointed out that type N patients requiring a series of transfusions at wide intervals should be transfused only with type N blood, if isosensitization and consequent transfusion reactions are to be avoided. Genetic and serologic analogies among the A-B-O, M-N and Rh-Hr systems of blood factors are pointed out. Evidence is cited to show that the factors in each system fall naturally into two major subdivisions.

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[10] DeKromme, L., and Van der Spek, L. A. M., *Brit. Med. J.*, 1948, **1**, 643.

16599

Cure of Ulcerative Cecitis of Rats by Streptomycin.

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Ulcerative cecitis of rats is a disease of considerable importance from two standpoints. First of all, when prevalent in a rat colony, experimental work carried out with the affected animals becomes confused, and incorrect conclusions may be drawn.

Secondly, the disease itself is of great interest. Since it is exquisitely chronic and interferes very little with the general health of the animals one has an excellent tool for the study of experimental epidemiology, the pathogenesis of infection and the effects of chemotherapy and antibiotics. Certain aspects of these questions have been dealt with in previous papers.[1-6]

In 1940-41 cecitis became prevalent in the Stanford rat colony. Very young animals were not affected, but by 4-5 months 44% of the stock had fresh lesions and the incidence increased thereafter so that 66% of 7-8 months rats showed lesions. The etiology of this interesting disease is not yet entirely clear. Cultures from the ulcerations have invariably yielded *Salmonella enteritidis*, but in spite of this we have raised the question of whether this organism alone is responsible. If bacteria of the Salmonella group freshly isolated from rats with cecitis are introduced into the drinking water of unaffected animals one produces a violent diffuse enteritis which in no way resembles the spontaneous disease. Rats which survive such an acute infection then proceed gradually to develop the usual lesions but with no greater frequency and to no greater extent than uninoculated controls.[3] Organisms, furthermore, have not been obtained from fluid aspirated from the cysts which so often develop around the cecum. It was also shown that the development of cecitis was inhibited in rats on a vitamin B deficient diet, a finding just the reverse of what one would expect with an ordinary bacterial infection.[6]

Be all this as it may, we found that cecitis could be prevented by incorporating 1% of succinyl sulfathiazole in the stock diet.[2] Sulfaguanidine 0.5% was equally effective.[1] The interesting observation was also made that if sulfaguanidine was added to the diet of the mothers during pregnancy and lactation the young subsequently failed to develop cecitis.[5] By these means the disease was eliminated from the colony and for the past three years no chemo-prophylaxis has been used.[4]

In the fall of 1947 there were indications that cecitis was again present in the colony, and the incidence was found to be as shown in Table I. The situation was even worse than in 1940 since the frequency in 4-5 months rats was now 83% as against 44% in the previous study. Furthermore, many of the animals showed very advanced lesions consisting of huge masses made up of glands, cysts containing clear fluid, omentum and thickened cecum. On opening the cecum the characteristic huge ragged ulcers were found. Cultures made from a number of the lesions all yielded bacteria of the Salmonella group in large numbers. These organisms showed the following reactions:

TABLE I.
Incidence of Cecitis in the Stanford Rat Colony, 1948.

Age of rats (days)	60	70-75	130-135
No. of rats	6	7	30
No. with cecitis	0	0	25
% with cecitis	0	0	83

glucose +
lactose —
saccharose —
inosite —
arabinose +
xylose +
dulcite +
mannite +
urea —
indol —

and they were considered to fall in the enteritidis group. Cultures from the cecums of several unaffected rats yielded no bacteria of the Salmonella group.

The sensitivity of the Salmonella bacilli isolated from cecitis was tested with streptomycin, and the organisms were found to be completely inhibited by 5 μg per cc of culture medium. In view of this finding the following observations were made to see if streptomycin had any therapeutic effect.

Experiment I. Twenty male rats were placed on the stock diet of the laboratory. On April 8, 1948, when the rats were about

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[1] Bloomfield, A. L., and Lew, W., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 363.

[2] Bloomfield, A. L., and Lew, W., *ibid.*, 1942, **51**, 28.

[3] Bloomfield, A. L., and Lew, W., *ibid.*, 1942, **51**, 179.

[4] Bloomfield, A. L., *ibid.*, 1943, **53**, 197.

[5] Bloomfield, A. L., and Lew, W., *Am. J. Med. Sc.*, 1943, **205**, 383.

[6] Bloomfield, A. L., and Lew, W., *J. Nutrition*, 1943, **25**, 427.

TABLE II.
Cecal Lesions Before and After Streptomycin Therapy.

Rat No.	Appearance at laparotomy May 11	Appearance at autopsy May 20 after streptomycin for 9 days
75275	Mass 1.5 cm with large glands and adherent omentum	Cecum slightly thickened. No open ulcer in cecum. A little thickening of tissues at hilum. Mass is gone.
75213	Mass 0.5 cm. Small adherent glands.	A tiny hard gland at hilum. Mucosa seems a little thickened. No ulcer
7400	Cecum itself is soft, but there is an adjacent mass of glands and cysts 1.5 cm in diameter	Small cecum with adhesions and tiny remains of a gland. Mucosa slightly thickened—no ulcer
74039	Mass 1.5 cm with large glands and cyst 0.5 cm. Much matting	A small mass of firm white glands in hilum. Adhesions to omentum. Mucosa seems thickened but no ulcer
74258	A 2 cm adherent mass of cysts and glands	Mass gone. Adhesions. Mucosa seems slightly thickened. No ulcer
75536	1.5 cm mass of glands and cysts	Cecum practically normal but thickening and adhesions at hilum with a small cyst containing fluid
75775	1.5 cm mass with glands and cysts. Omentum adherent	Mucosa normal. Some scarring and adhesions outside with small hard hilar nodes
76191	Great pericecal adhesions with cyst and small mass in cecum	Cecum scarred and adherent. A large cyst containing fluid. Mucosa slightly thickened—no ulcer
76199	Mass 0.5 cm with adherent omentum and glands	Pericecal adhesions. Mucosa normal—no ulcer
77435	Huge mass nearly 3 cm in diameter. Everything matted and adherent	Remarkable resolution. Many adhesions but no glands. Mucosa much thickened. A gray area nearly 1 cm in diameter marks remains of what must have been a large encrusted ulcer before treatment
76498	0.5 cm adherent mass of glands and cysts	*No Streptomycin.* Thickened adherent cecum. Huge 2x2 cm typical green ulcer in usual situation in cecum.

130 days old one-half the group were sacrificed and the incidence of cecitis was noted. All but one of the group had definite lesions. One might expect, therefore, a similar incidence of cecitis in the remaining 10 rats. On April 12th streptomycin was dissolved in the drinking water of these animals so that each rat took on the average of 0.1 g of the antibiotic daily. The rats throve on the streptomycin and soon disdained plain water. During the 10 days of streptomycin therapy all the animals gained weight, some as much as 20 to 25 g. On April 22nd the rats were killed. In contrast to the controls sacrificed 10 days previously none of the treated animals had definite lesions with the exception of one rat which showed the faint remains of a shallow ulcer 0.5 cm in diameter and obviously healing. In rat 12 the cecum appeared normal but a slight glandular enlargement still remained at the hilum. Cultures from the cecums of five rats yielded no growth, and sections through the entire organ showed no active ulcerations except in one case in which there was a small acute shallow ulcer with complete erosion of the mucosa. However all the cecums showed infiltration of the submucosa with eosinophiles and to a lesser extent neutrophiles, evidently the remains of a healing process.

Streptomycin appears then to have a striking therapeutic effect in ulcerative cecitis of rats. However, it seemed necessary to control our observations even more precisely. The following experiment was therefore carried out:

Experiment II. A number of rats in which a clinical diagnosis of cecitis could be made on the basis of a palpable abdominal mass were collected. On April 11th, exploratory laparotomy was done on 11 animals under ether anesthesia and the condition of the cecum was carefully noted (see Table II). The incisions were closed and streptomycin was immediately added to the drinking water of 10 rats so that each animal took on the average 0.1 g daily. The remaining animal received no antibiotic. After 9 days all the animals were sacrificed. The results are shown in Table II. As in Experiment I a remarkable therapeutic effect is observed. While there were some residues of infection in the way of scarring and adhesions, not a single open ulcer was found in the treated rats. The untreated animal (No. 76498) on the other hand showed the typical changes of active cecitis with a large ulcer.

Summary. The lesions of ulcerative cecitis of rats resolved within 10 days of streptomycin treatment. The antibiotic was added to the animals' drinking water in an average dose of 0.1 g per day per rat. The bacilli of the Salmonella group which are invariably associated with the ulcers of cecitis were sensitive to streptomycin in the test tube and they could no longer be grown from the cecums of treated rats with healed lesions.

16600 P

Simple Intestinal Obstruction: Prolongation of Life for 45 Days with Parenteral Alimentation.

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(Introduced by I. S. Ravdin.)

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The average length of survival of animals having a low simple obstruction of the ileum when allowed fluids by mouth or when given saline parenterally is around 8 to 14 days.[1,2] Death has been attributed to loss of fluid and electrolytes, possible nervous effects of distension, or the absorption of toxins from the obstructed bowel. By administering fluids, electrolytes, and nutritive material into an enterostomy below a high simple intestinal obstruction, Jenkins[3,4] has maintained animals for from 50 to 70 days. No cause for death other than the long standing emaciation was found.

We have studied the course and length of survival of animals having a simple obstruction of the ileum in which electrolyte imbalance, dehydration, anemia, and starvation were combatted by the intensive administration intravenously of sodium chloride, 5 or 10% glucose, casein hydrolysate,* blood, plasma, gelatin, and vitamins in an effort to see if by parenteral administration obstructed animals could be maintained as long as those fed by enterostomy.

Method. Ten healthy adult dogs weighing between 10.0 and 15.0 kg were used. The mid-ileum was obstructed by severing the bowel and closing the ends. Postoperatively fluids were administered daily either by intermittent rapid drip intravenously with the animal on the table or, in the later experiments, by constant intravenous drip through a capillary plastic tube threaded into the jugular vein or inferior vena cava after the method of Rhode, Parkins and Vars,[5] except that a deBakey pump was not used. The amounts of fluids and calories to be administered were determined by the output (urine and vomitus) and chemical studies on the blood, but it was not always possible to meet the requirements because of the limitations of the methods used for administration; namely, the length of time the animal

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[1] Harper, W. H., and Lemmer, R. A., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 207.

[2] Elman, R., and Hartman, A. F., *Surg., Gynec. and Obst.*, 1931, **53**, 307.

[3] Jenkins, H. P., PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 111.

[4] Jenkins, H. P., and Beswick, W. F., *Arch. Surg.*, 1933, **26**, 407.

* Amigen 5% in 5% dextrose solution made by Mead Johnson & Company.

[5] Rhode, C. M., Parkins, W. M., and Vars, H. M., *Bull. Am. Coll. Surg.*, 1947, **32**, 255.

would remain on the table, thrombosis of veins, dislodgement of the plastic tube, and inconstancy of rate of flow. Water and dog biscuits were placed in the cages of the animals receiving fluids intermittently but not in the cages of those receiving constant parenteral alimentation.

Results. Five of the 10 animals died between 2 and 6 days, 2 of electrolyte imbalance, 1 of intussusception with gangrene and necrosis of the distal turned-in end, 1 of leakage of the suture line with a resulting peritonitis, 1 of distemper. The remaining 5 lived between 16 and 45 days with an average survival time of 27.6 days. One died suddenly at 16 days, and necropsy revealed an intussusception of the distal turned-in end with gangrene and necrosis. Another was reoperated at 21 days, while in good condition, and the intestinal continuity restored. The remaining 3 dogs survived for 24, 32, and 45 days respectively, and death was due to emaciation and resulting complications.

That the length of survival was closely correlated with the adequacy of parenteral alimentation was indicated by the animal which lived for 45 days. This animal was maintained in electrolyte balance throughout the course of survival. The average daily intake was 1,091 cc and output 722 cc. Hypoproteinemia and anemia became marked around the fifth and sixth weeks. The animal lost 26% of the initial body weight and was extremely emaciated at death. For the first 10 days, due to much technical difficulty with the plastic tubing, an average of 140 cc of milk daily was allowed and taken orally. Thereafter nothing was allowed by mouth except 10.0 cc of a vitamin mixture daily.

It will be noted (Table I) that only during the last 15 days were we able to administer parenterally sufficient nitrogen and calories to even approach a level adequate to maintain nitrogen balance,[6] and even at this level the animal continued to lose an average of 0.7% of the body weight daily. However,

TABLE I.

		Avg daily								
Dog No. 10	Time	Intake, cc	CHO, g/kg	N, g/kg	cal./kg	NaCl, mg	Output, cc	Weight loss, kg	Weight loss, %	Temp.
Length of survival 45 days	First 10 days	881	4.36	0.14	22.45*	4.81	396	0.06	0.4	1024
	Second 10 days	958	5.15	0.18	23.86	5.58	745	0.12	0.8	1030
	Third 10 days	1,230	7.20	0.23	32.68	7.55	834	0.07	0.5	1026
	Last 15 days	1,226	7.76	0.33	37.20	6.78	850	0.09	0.7	1030

* Includes calories from fat in milk administered for first 10 days.

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[6] Riegel, C., Koop, C. E., Grigger, R. P., Rhoads, J. E., and Bullitt, L., *S. Clin. N. Am.*, 1945, 1096-1105.

despite the inability to maintain adequate nutrition, and in the face of intermittent, severe vomiting (1500 to 2500 cc every 3-4 days) and a sustained elevated temperature due to thrombosis of veins resulting from the methods used, this animal was maintained for 45 days, and during the last 35 days all fluids were administered intravenously.

Conclusions. The length of survival of dogs having a low simple intestinal obstruction depends upon the ability to maintain fluid, electrolyte, and nutritional requirements. By parenteral alimentation with the methods used adequate nutritional requirements were not obtained, but in 1 animal in which these requirements were approached more closely the length of survival was 45 days.

16601 P

A Rapid Mouse Test for Laboratory Diagnosis of Tuberculosis.*

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Recent reports have shown that pigmented strains of mice (dba, C3H and C57 black) are more susceptible to tuberculosis than albino mice.[1,2] We have confirmed these findings and have completed additional experiments in which albino and pigmented mice were inoculated intraperitoneally with tubercle bacilli suspended in various diluents. This was done with the hope of finding an enhancing agent for a rapid diagnostic test for tuberculosis.

This paper is a preliminary report on the use of gastric mucin for enhancing the virulence of the H37Rv and several recently isolated strains of mammalian tubercle bacilli for pigmented mice. Although there have been no previous reports to our knowledge on the inoculation of mice with mucin suspensions of tubercle bacilli, Mills and Colwell[3] showed that guinea pigs inoculated subcutaneously with small doses of tubercle bacilli suspended in mucin developed larger local lesions than controls inoculated with saline suspensions. These workers found no difference in pathogenicity when larger dosages were inoculated. More recently Pierce *et al.*[2] have shown that the infectivity of tubercule bacilli for mice is enhanced by egg yolk.

For the present studies CFW albino mice and C3H, dba and C57 black strains of pigmented mice weighing about 13 g were used. The virulent human type tubercle bacillus H37Rv or recently isolated strains from cases of tuberculosis were studied. Suspensions were prepared from 3-4-week-old cultures grown on Petragnini medium. The organisms were suspended in physiological saline and clumps were dispersed by means of glass beads. The suspension was adjusted to approximately 1 million organisms per cc by a nephelometer tube, and the count was checked with a Petroff-Hausser counting chamber. Mucin was prepared by suspending 5 grams of granular mucin (Type 1701-W, Wilson lab.) in 100 cc of distilled water, adjusted to pH 7.4 and autoclaved. Egg yolk was obtained aseptically and diluted with equal parts of physiological saline.

Results comparing the virulence of the H37Rv strain suspended in mucin, egg yolk

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* Aided by the Sheila Kelman Memorial Fund. Supported in part by the Michael Reese Research Foundation. We are indebted to Sylvia King and Phyllis Conner for technical assistance.

[1] Long, E. R., and Vogt, A. B., *Am. Rev. Tuberculosis,* 1941, **44**, 196.

[2] Pierce, C., Dubos, R. J., and Middlebrook, G., *J. Exp. Med.,* 1947, **86**, 159.

[3] Mills, M. A., and Colwell, C. A., *Am. Rev. Tuberculosis,* 1939, **40**, 109.

TABLE I.
Effect of Gastric Mucin, Egg Yolk, and Saline Diluents on Virulence of Tubercle Bacilli (H37Rv) for C3H and CFW (Albino) Mice.

Mouse strain	Diluent	No. of mice	No. with macroscopic lesions	% liver lesions	% peritoneal and lymph node lesions
C3H	Mucin	18	18	39	88
,,	Egg yolk	18	10	40	60
,,	Saline	16	0	0	0
CFW	Mucin	15	2	0	100
,,	Saline	12	0	0	0

and saline for C3H and CFW (albino) mice are summarized in Table I. The mice were inoculated intraperitoneally with 0.5 cc of each mixture composed of equal parts of tubercle bacilli suspension and diluent. Each mouse was inoculated with approximately 500,000 bacilli. All animals were sacrificed 10 days after inoculation and autopsied for evidence of macroscopic lesions. Impression smears were routinely made of gross lesions and stained by the Ziehl-Neelsen technic. Lesions varied in size from 2 to 3 mm and usually contained large numbers of tubercle bacilli in impression smears. When present lesions were seen at the site of inoculation, liver, spleen or intestinal lymph nodes. A few tubercle bacilli were usually found in impression smears of the spleen or liver of mice inoculated with mucin suspensions, even if macroscopic lesions were not present in these organs. No gross lesions were seen in the lungs. Similar results were obtained using dba mice and recently isolated tubercle bacilli.

Since the above experiments were completed we have made several (14) isolations of tubercle bacilli from patients 10 to 15 days after inoculation by the use of pigmented mice (C3H or dba) and mucin. A few of the positive results in mice were obtained with microscopically negative sputum. The patient's sputum or feces were prepared in the usual manner, mixed with equal parts of 5% mucin, and inoculated both subcutaneously and intraperitoneally (0.5 cc) into each of 4 or 5 mice. Tubercle bacilli were readily recovered from a number of the lesions planted in Petragnini's medium. Impression smears of the spleen, liver and peritoneal fluid were routinely made of all inoculated mice because acid-fast bacilli could sometimes be demonstrated although no macroscopic lesions were present. Comparative studies are now in progress in which routine specimens from patients are being inoculated into mice, guinea pigs and culture media simultaneously.

Summary. Gastric mucin significantly enhances the virulence of tubercle bacilli for pigmented strains (dba, C3H or C57 black) of mice. Grossly visible lesions containing large numbers of tubercle bacilli are usually present 10 days after intraperitoneal inoculation with the dosages and strains of tubercle bacilli studied. Several isolations of tubercle bacilli from patients have been made 10 to 15 days after inoculation by use of pigmented mice (dba or C3H) and mucin.

Addendum. Recently the number of isolations from patients has been increased by feeding pigmented mice inoculated with mucin the cornmeal-gelatin-butter diet of Dubos and Pierce.[4]

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[4] Dubos, R. J., and Pierce, C., *Am. Rev. Tuberculosis*, 1948, **57**, 287.

16602 P

Histologic Evidence for the Synthesis of Protein in Lymphocytes Following Parenteral Injection of Antigen.

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Experimental evidence for the formation of antibodies in the local lymphatic system following the parenteral injection of antigen was presented by McMaster, *et al.*,[1,2] and by Ehrich and Harris.[3] Dougherty, Chase and White[4] demonstrated antibodies in lymphocytes, and Harris, *et al.*,[5] showed that lymphocytes were a primary site of antibodies following the local injection of antigens. Other cells of mesodermal origin have, however, been regarded as the possible sources of antibodies: macrophages[6] and, more recently, plasma cells.[7,8]

The recent applications of histochemistry to physiology have offered an additional approach to this problem. It was first shown by Caspersson[9,10] and Brachet[11] that cells which were actively forming new protein were characterized by large amounts of ribonucleic acid in their cytoplasm. Additional evidence for the association of cytoplasmic ribonucleoprotein with protein synthesis has been reviewed by Greenstein[12] and by Dempsey and Wislocki.[13] Since there is considerable immunologic evidence of the production of antibody by the popliteal lymph node of the rabbit[3,14,15] following the injection of antigens in the foot pad, and since a tissue in which antibody is being formed could be expected to show evidences of protein synthesis, it seemed feasible to attempt to identify the cells involved by histochemical methods.

Accordingly, a study was undertaken of the localization and concentration of ribonucleic acid in the cells of the popliteal lymph node, in correlation with the production of antibody by that node, after the injection of antigens into the local tissue. This preliminary report will present a brief description of the histologic changes in the lymph node during the first few days after the injection of the antigen.

Methods. Injections of influenza virus into the pads of rabbit feet and the collection of regional (popliteal) lymph nodes were made as described elsewhere.[15] Each animal in this series received the antigen only in one foot, so that the contralateral lymph node could serve as a control specimen. Animals were sacrificed at 1, 2, 3 and 4 days after the injection of antigen. Both popliteal lymph nodes were excised. Parallel sections of each node were stained by Azur II-Eosin and methyl-green-pyronine respectively. The latter set of stains is for the respective nucleic acids: methyl green stains desoxyribonucleoprotein a somewhat bluish green, and pyronine stains ribonucleoprotein red.

Results. The histological findings in the Azur-Eosin stained sections of these popliteal

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[1] McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1935, **61**, 783.

[2] McMaster, P. D., and Kidd, J. G., *J. Exp. Med.*, 1936, **66**, 73.

[3] Ehrich, W. E., and Harris, T. N., *J. Exp. Med.*, 1942, **76**, 335.

[4] Dougherty, T. F., Chase, J. H., and White, A., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 295.

[5] Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.*, 1945, **81**, 73.

[6] Sabin, F. R., *J. Exp. Med.*, 1939, **70**, 67.

[7] Bjoerneboe, M., Gormsen, H., and Lundquist, F., *J. Immunol.*, 1947, **55**, 121.

[8] Fagraeus, A., *J. Immunol.*, 1948, **58**, 1.

[9] Caspersson, T., *Naturwissenschaften*, 1941, **29**, 33.

[10] Caspersson, T., *Chromosoma*, 1941, **1**, 605.

[11] Brachet, J., *Arch. Biol. Paris*, 1942, **53**, 207.

[12] Greenstein, J. P., *Advances in Protein Chemistry*, 1944, **1**, 210.

[13] Dempsey, E. W., and Wislocki, G. B., *Physiol. Rev.*, 1946, **26**, 1.

[14] Ehrich, W. E., and Harris, T. N., *J. Exp. Med.*, 1946, **83**, 373.

[15] Harris, S., and Harris, T. N., in press.

lymph nodes have been reported elsewhere[15] and will not be repeated here. On examination of the sections of normal nodes by methyl-green and pyronine, almost no pyronine stained material could be found.

The histologic differences among the stimulated nodes were primarily those of degree. In these nodes, one day after the injection of antigen, a few young lymphocytes were seen to contain red granules in the cytoplasm, and in many of these cases, clearly defined red nucleoli. Such cells were progressively more numerous on the second, third and fourth days. On these latter days there was an occasional crescentic accumulation of pyronine-staining material at the very edge of the nucleus of cells which showed this material, and occasionally cells were seen which showed no cytoplasm except for a sector of material solidly stained red, contiguous to about one-quarter of the circumference of an otherwise denuded nucleus.

No plasma cells were seen in the greatly enlarged cortex of the stimulated lymph nodes by either method of staining. The few plasma cells noted were in the medullas of lymph nodes, which were fairly similar in area to those of control nodes, and the number of these cells did not differ remarkably, in these preparations, between control and stimulated nodes.

The great majority of cells with pyronine-staining granules were young lymphocytes. A few more mature, smaller, lymphocytes showed these granules, on the third and fourth days, and a number of the cells with these granules had nuclei so vesicular as to suggest that they were transitional forms between the reticulum cells on one hand and clearly recognizable young lymphocytes on the other. Within this range the distribution was continuous: A complete series of cells with pyronine-staining cytoplasmic granules could be picked out of a given section, from transitional forms to mature lymphocytes. Large lymphocytes of various stages were certainly much more in evidence than the mature cells among those with ribonucleic acid granules.

It is well to recall that the pyronine-staining granules are not the new protein, or antibody, itself, but a part of the mechanism which produces this protein. Accordingly it is quite consistent with our interpretation that the ribonucleic acid granules are found primarily in young rather than old lymphocytes.

Summary. The identification of the cell within lymphatic tissue which produces antibody has been approached by the application of a new histochemical technic for localization of cytoplasmic ribonucleic acid. The latter has been found to be an invariable concomitant to the formation of new protein in tissue. In lymph nodes actively engaged in the production of antibodies a wide range of lymphocytes, largely younger forms, was found to have cytoplasmic granules and nucleoli stained with pyronine, which is used to identify ribonucleic acid. Such granules and nucleoli were found also in transitional forms between reticulum cells and lymphocytes, but in no other cell types.

16603

Viscosity Studies of Erythrocytes from Persons with Sickle Cell Disease.

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The viscosity of the erythrocytes of sickle cell disease varies with changes in oxygen tension as Ham and Castle[1,2] have shown. The variation in viscosity is due to changes in

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[1] Ham, T. H., and Castle, W., *Trans. Assn. A. Phys.*, 1940, **55**, 127.

[2] Ham, R. H., and Castle, W., *J. Clin. Invest.*, 1940, **19**, 788.

the red cell itself and not to influences of the suspending medium.[3]

It was the purpose of this study to investigate the possibilities of the viscosimetric method as as objective means of measuring the sickling tendency of the red cells of individuals or groups of individuals under varying conditions.

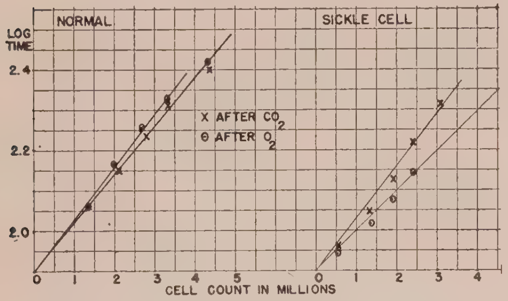

FIG. 1.

Log. of time of flow in seconds plotted against red cell count in millions, after exposure to O_2 and CO_2.

Methods. An Ostwald viscosimeter with a capillary bore of 0.48 mm in diameter and a length of 7.18 cm was used. The upper reservoir of the viscosimeter was calibrated at a volume of 2 ml. The viscosimeter was suspended in a water bath at 37° C.

Freshly drawn oxalated blood was centrifuged and the erythrocytes washed 3 times in Tyrode's solution. Three ml of cell suspension in Tyrode's solution was placed in the lower reservoir. The appropriate gas was passed through the suspension to effect cell saturation. The time of flow through the capillary was measured with a stop watch.

Results. In Fig. 1 the viscosities of various cell suspensions from a normal subject (No. 1) and from a subject with the sickle cell trait (No. 7) are shown. Viscosity, expressed as the logarithm of the time in seconds, is plotted against the cell count in millions.

Tables I-VI show the results of various studies of blood subjects with sickle cell disease.

TABLE I.

Viscosimeter Time Intervals and Cell Counts for Red Cell Suspensions.

Subject	After O_2	After CO_2	Time difference in seconds	Cell count in millions
1 (Normal)	217	207	— 10	3.9
2 ”	233	229	— 4	3.5
3 ”	152	151	— 1	2.3
4 ”	262	253	— 9	4.5
5 ”	271	259	— 12	4.3
6 ”	244	228	— 16	4.6
7 (S. C. T.)	192	272	+ 80	4.4
8 (S. C. T.)	236	498	+262	3.8
9 (S. C. T.)	197	321	+124	3.1
10 (S. C. T.)	132	151	+ 19	2.0
11 (S. C. T.)	150	185	+ 35	2.4
12 (S. C. A.)	182	267	+ 85	2.2
(A) (Year later)	139	200	+ 61	2.0
13 (S. C. A.)	141	157	+ 16	2.1
(A) (Year later)	139	200	+ 61	2.0
14 (S. C. A.)	189	281	+ 92	4.2
15 (S. C. A.)	152	175	+ 23	2.1
16 (S. C. A.)	202	320	+116	2.5

TABLE II.

Effect of Nitrogen

	Time in seconds		
Subject	After O_2	After N_2	After CO_2
17 (S. C. A.)	152	182	186

Discussion. Several methods for the determination of the viscosity of blood have been used.[4,5] Both the capillary method and the concentric ring method indicate that the

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[3] Huck, J. G., *Johns Hopkins Hosp. Bull.*, 1923, **34**, 335.

[4] Fahreus, R., and Lindquist, T., *Am. J. Physiol.*, 1931, **96**, 562.

[5] Brundage, J. T., *Am. J. Physiol.*, 1934-1935, **110**, 659.

TABLE III.
Effect of Change of pH. Phosphoric Acid Added.

Subject	After O_2		After CO_2		Cell count in millions
	pH	Time in secs.	pH	Time in secs.	
18 (S. C.A.)	7.8	160	6.35	221	2.5
Acid added	6.25	138	6.10	192	2.0

TABLE IV.
Effect of Carbon Monoxide.

Subject	Time in secs.			Cell count in millions
	After O_2	After CO_2	After CO	
10 (a)	132	147	133	2.0
(b)	106	112	107	1.4

TABLE V.
Effect of Repeated Washing of Cells with Tyrode's Solution and Normal Saline.

Subject	Solution	Time in secs.		Cell count in millions
		After O_2	After CO_2	
19 (S. C. A.)	Tyrode's, 3 ×	160	195	1.9
	Tyrode's, 20 ×	112	130	1.7
	Normal saline, 20 ×	119	134	2.4
	On ice—24 hr			
	Normal saline, 20 ×	111	120	1.2

TABLE VI.

Subject	Time in secs.		Cell count in millions
	After O_2	After CO_2	
20 (a)			
S. C. A. (Heparin)	128	145	2.3
(b) (Oxalate)	123	140	2.0
(c) (NaCN added to *b*)	126	140	2.0

apparent viscosity of blood will vary with the procedure used for its determination. The size of the capillary and variations in pressure are important in the capillary tube method. The speed of rotation is an important variable in the concentric ring method. In this particular investigation an Ostwald viscosimeter with a relatively wide bore was used so that time intervals would be short and there would be a minimum of sedimentation of red cells. Actual viscosities were not calculated and differences are indicated by varying time intervals.

The viscosity of blood depends upon a number of factors related to the composition of blood itself, most important of which are the protein content of the plasma and the number and character of the erythrocytes. Red cells separated from plasma and resuspended in Tyrode's solution display viscosity changes due only to changes in the characteristics of the red cells.

Changes in viscosity of suspensions of red cells from persons with sickle cell disease are easily demonstrable and are compared in Table I with normal cell suspensions. It will be seen that such viscosity changes occur in cell suspensions from subjects with sicklemia (S.C.T.) as well as from subjects with sickle cell anemia (S.C.A.)

There are compared, in Fig. 1, the viscosity measurements of cell suspensions from normal individuals and subjects with sickle cell disease, after exposure to oxygen and to carbon dioxide. It is shown that the viscosity of sickle cells differs from that of normal cells after treatment with carbon dioxide. Increase in viscosity with nitrogen is shown in Table II.

Variations of the hydrogen ion concentration do not seem to affect the viscosity of the abnormal cells (Table III).

Carbon monoxide reacts with hemoglobin in much the same way as oxygen although a far more stable compound, carbon monoxide hemoglobin, is formed. Murphy and Shapiro[6] found that exposure of cells from a subject with sickle cell anemia to carbon monoxide apparently prevented their sickling. In order to confirm this, cells which had the sickling tendency were treated with carbon monoxide and studied in the viscosimeter. The sickling process was inhibited by this treatment as it was no longer possible to produce viscosity changes with carbon dioxide or nitrogen (Table IV).

Tomlinson and Jacob[7] report an inhibition of sickling by repeated washings and by the addition of cyanides to sickle cell blood. It could not be demonstrated by the viscosimetric method that repeated washings inhibited the sickling tendency (Table V). The addition of cyanide or the use of various anticoagulants showed no influence on the sickling tendency as viscosity changes could still be demonstrated after their use (Table VI).

Summary. A viscosimetric method is described for the study of the sickling tendency of red cells from subjects with sickle cell disease. This method does not differentiate between sickle cell anemia and sickle cell trait. Data are compared with observations of other investigators.

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[6] Murphy, R. C., and Shapiro, S., *Ann. Int. Med.*, 1945, **23**, 376.

[7] Tomlinson, W. J., and Jacob, J. E., *J. Lab. and Clin. Med.*, 1945, **30**, 107.

16604 P

Studies of Typhus Fever in Peiping by the Complement Fixation Test.

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Recently, the complement fixation reaction in typhus fever, first studied by Castaneda,[1] has been refined so that not only typhus may be differentiated from other rickettsial diseases such as Rocky Mountain Spotted Fever,[2] but may itself be classified according to epidemic or murine nature.[3] Inasmuch as we have been interested in the etiology of typhus fever in this area, and have reached certain tentative conclusions based on the studies of the biology of the rickettsia strains locally isolated,[4] we wish now to use the complement fixation test to complete our studies. Unfortunately so far, owing to the scarcity of clinical cases seen in the past year, the number of sera examined has been small. But the results seem to be sufficiently consistent to warrant a preliminary report at this time.

Method and results. The antigens used in the present study, kindly supplied by the Lederle Laboratory Division of the American Cyanamid Company,[5] consist of washed rickettsial bodies. The method of complement fixation test is that of Kolmer's modified by reducing all ingredients to one-half the volume of the regular method. Sera were obtained from patients suffering from clinical typhus fever[6] some of which have been preserved by the lyophile process. Any serum showing anticomplementary action was treated by the method of Faran.[7] It was found that all except one gave positive reaction to both antigens, with titres mostly above 1:320, and one reaching as high as 1:20,480. Among these, 5 sera showed the same titre for both antigens, while 14 sera from 10 patients showed a higher titre to the epidemic antigen: 4 with a 2-fold difference, 4 with 4-fold difference, 5 with 8-fold difference and one positive only to epidemic antigen at titre 1:640.

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[1] Castaneda, M. R., *J. Immunol.*, 1936, **31**, 285.

[2] Bengtson, I. A., *Am. J. Pub. Health*, 1945, **35**, 701.

[3] Wishart, F. O., and Malcomson, M. E., *Canad. Pub. Health J.*, 1946, **37**, 369.

[4] Liu, W. T. *et al.*, *Am. J. Hyg.*, 1942, **35**, 231.

[5] We are indebted to Dr. R. B. Watson, of the International Health Division of the Rockefeller Foundation for securing these antigens.

Comment. The results of the present study by means of complement fixation test agree with a similar study, carried out by one of us (N.C.C.) in the spring of 1946 when epidemic and murine vaccines served as antigens. In that study, 9 patients with 15 samples of sera gave clear cut positive complement fixation reaction with one or both of the antigens. Seven patients with 10 sera gave stronger reaction to the epidemic, one gave equal titre, while another one gave higher titre to the murine antigen. These findings seem to suggest that the strains of rickettsia causing human infections in the past 2 years, whether in the autumn or in the spring, are largely of the epidemic type. This is in contrast to our previous results when the biological study of the organisms tended to classify them as murine type. In order to reconcile the above differences, further study will be made with perhaps absorbed sera. These will be continued when sera from fresh typhus cases will be available.

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[6] We are indebted to Dr. Y. W. Yih, of the Hospital of the National Pei Ta Medical School for 4 of the sera tested.

[7] Faran, A., *J. Lac. Clin. Med.*, 1946, **31**, 1037.

[8] Bengtson, I. A., *Pub. Health Rep.*, 1947, **61**, 1379.

16605

Isometric Relaxation Period of the Left Ventricle in Normal Subjects and Patients with Mitral Stenosis.

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Differentiation of a split second sound from reduplication of this sound is important; the latter is consequent to loud snapping of the mitral leaflets in mitral stenosis while the former has a variety of causes, the most common of which is asynchronous closure of the aortic and pulmonic valves. Important information bearing on this problem may be afforded by study of the duration of the period of isometric relaxation of the left ventricle, for this period is the interval between closure of the aortic valve and opening of the mitral. Several attempts have been made to measure this period in normal subjects by means of arterial and venous tracings[1] or by observation of roentgenological tracings (electrokymograms).[2] The phonocardiogram has been used, thus far, for measuring this interval only in patients with mitral stenosis.[3] Errors due to delay in the transmission of the v wave from the right heart to the neck or asynchronism between right and left ventricles cannot be avoided in studies based on mechanical methods; similarly, inaccuracies due to the influence of position and motion phenomena may be present in roentgenological tracings. However, the phonocardiogram gives a virtually instantaneous recording of the vibration due to valvular movements and is

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[1] Burstein, J., *Am. J. Phys.*, 1923, **65**, 158.

[2] Randak, E. F., Boone, B. R., Ellinger, G. E., and Oppenheimer, M. J., *Fed. Proc.*, 1948, **7**, 97.

[3] Margolies, A., and Wolferth, C., *Am. Heart J.*, 1932, **7**, 443.

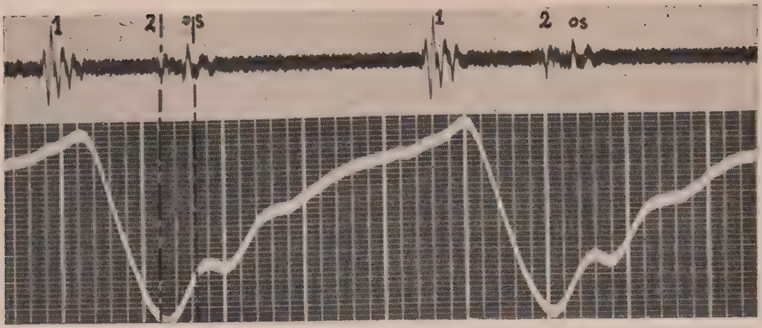

Fig. 1.

Simultaneous phonocardiogram and fluorocardiogram in a normal subject. The former has two vibrations indicating the closing of the semilunar valves (2) and the opening of the mitral valve (os). Between them lies the isometric relaxation period.

independent of other causes of error; it is therefore, the most accurate method. Thus far, only data obtained in cases of mitral stenosis are available[3] because, even if recognition of the various components of the second sound is often possible,[4] the vibration due to opening of the mitral valve can be recognized only in a small number of normal subjects. However, the systematic study of a series of over 1500 phonocardiograms collected by one of us (Luisada) showed the possibility of clearly isolating the vibration due to the opening of the mitral valve in a small percentage of cases. This vibration will be called "opening sound of the mitral valve".

Materials and Methods. This study is based on the above mentioned collection of phonocardiograms as well as the collection of fluorocardiograms and phonocardiograms of the Beth Israel Hospital. The records of seven normal subjects permitted measurement of the isometric relaxation period of the heart on the basis of the phonocardiogram. One of these cases also had a fluorocardiogram (electrokymogram) recorded and, therefore, permitted the analysis of the latter tracing on the basis of the phonocardiographic study (Fig. 1). Twenty-four cases had mitral stenosis with an evident opening snap of the mitral valve. This permitted the determination of the isometric relaxation period of the left ventricle in rheumatic heart disease where there is a possibility of delayed opening of the left auricular-ventricular valve. Parallel studies were made in 9 cases of mitral stenosis with a split second sound at the base, in 19 subjects having a similar splitting without mitral stenosis, and in 20 cases with a third heart sound. The phonocardiograms were recorded by means of a Sanborn "Stethocardiette", using a stethoscopic microphone with a 3.7 cm funnel applied at the apex, at the third left interspace, and at the second left interspace. The fluorocardiogram is a tracing of the movements of the cardiovascular fluoroscopic silhouette recorded by a phototube and a galvanometer. Details of this method have been given pre-

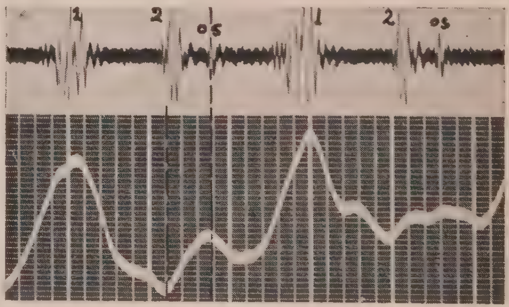

Fig. 2.

Simultaneous phonocardiogram and fluorocardiogram of a patient with mitral stenosis and auricular fibrillation. The sound tracing presents a loud opening snap of the mitral valve (os). Between the main vibration of the second sound (2) and the opening snap (os) is the isometric relaxation period which is prolonged.

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[4] Rappaport, M. B., and Sprague, H. B., *Am. Heart J.*, 1942, **23**, 591.

TABLE I.

	Distance between main phase of 2nd sound and opening sound (or snap) of the mitral valve		Distance between the two phases of the split 2nd sound	Distance between 2nd and 3rd sounds
	Normal subjects	Mitral stenosis		
Maximum	0.07	0.11	0.05	0.18
Minimum	0.04	0.07	0.025	0.12
Average	0.055	0.079	0.04	0.15

viously.[5,6,7] In our collection of fluorocardiograms, 32 tracings were recorded in patients with mitral stenosis; one of the latter had a pronounced snap of the mitral valve (Fig. 2).

Results. The results are presented in Table I, in seconds.

The normal subject presenting a visible opening sound in the phonocardiogram, on whom a fluorocardiogram was recorded, showed the following:

(a) The interval between the main vibration of the second sound and the opening sound was 0.07 seconds.

(b) The tracing of the ventricle exhibited an early diastolic rebound.

(c) While the negative peak of the ventricular tracing coincided with the main vibration of the second sound, the opening sound coincided with the end of the initial rise and preceded the rebound (Fig. 1). Similar findings were present in tracings recorded along the left ventricular margin.

The patient with mitral stenosis and an opening snap of the mitral valve on whom a fluorocardiogram was recorded showed the following:

(a) The interval between main vibration of the second sound and opening snap was 0.11 second.

(b) The tracing of the left ventricle presented an early diastolic rebound.

(c) The relationship between opening snap and rebound is identical with that of the normal subject in spite of the prolongation of the isometric relaxation period.

Discussion. Our average figures for the duration of isometric relaxation in normal subjects are somewhat less than those found by Burstein.[1] This can be explained by the greater accuracy of our method which excluded transmission time of mechanical waves from the heart to the neck and results of phenomena taking place in both ventricles; moreover, our data were obtained by the use of a speed film. On the other hand, our findings coincided with those reported by Hoff.[8] Our data concerning measurements in patients with mitral stenosis are similar to those of Margolies and Wolferth[3] in regard to the average figures. Their minimum and maximum figures are respectively smaller and larger than ours, but they were found during extreme alterations of the cardiac rate. Our data on the interval between the two phases of a split second sound are shorter than those of Wolferth,[9] possibly because of a different choice of cases, while those concerning the third sound are identical with his. There is a slight difference between our data concerning the third sound and those of Rappaport and Sprague.[4] This difference can be explained by the fact that these authors measured the interval from the beginning of the second sound.

Simultaneous observation of the phono-

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[5] Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, **35**, 336 and 348.

[6] Luisada, A. A., and Fleischner, F. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 436.

[7] Fleischner, F. G., Romano, F. J., and Luisada, A. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 535.

[8] Hoff, H. E. Events of Cardiac Cycle. In Howell's Textbook of Physiology by Fulton, Saunders, Philadelphia. 1946.

[9] Wolferth, C. C. Heart Sounds, in Cyclopedia of Medicine, Vol. 3. F. A. Davis, Philadelphia.

cardiogram and fluorocardiogram, both in the case with a physiological opening sound and in that with a pathological opening snap, indicates the following:

The part of the fluorocardiogram which corresponds to the isometric relaxation period lies between the lowest point of the ventricular wave and the beginning of the rebound, and not that ending with the lowest point of the rebound, as suggested by others.[10] However, this period cannot be measured routinely on the various fluorocardiographic records because the diastolic rebound is not a constant feature; it may be represented by a less steep rise of the tracing or may be completely absent. According to our data, the figures found recently by others[2] for the isometric relaxation period are too long, especially because they did not study patients with mitral stenosis; as known, these may present the longest duration of the isometric relaxation period because of impaired opening of the mitral valve.

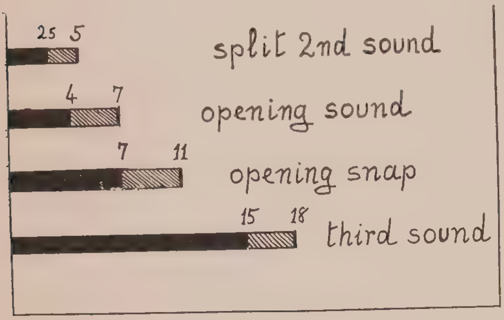

Fig. 3.

Scheme indicating the distance in hundreds of seconds between the two phases of a split or reduplicated second sound and between second sound and third sound. The black bar indicates the minimal distance, the striped bar, the maximal distance between the two phases of the reduplicated sound or between second and third sound. *Split second sound,* recorded over the pulmonic area. *Opening sound,* recorded over the mitral area in normal subjects. *Opening snap,* recorded over the apex or mitral area in patients with mitral stenosis. *Third sound,* recorded over the apex in normal subjects.

The results of our study, sketched in Fig. 3, facilitate basing the classification of an extra-sound on the above temporal data.

Summary. The isometric relaxation period of the left ventricle in normal subjects and in patients with mitral stenosis was studied. This period was measured on the phonocardiogram as the interval between the main vibration of the second sound and the opening sound or snap of the mitral valve.

The findings in 7 normal subjects varied between 0.04 and 0.07 seconds; 24 mitral patients had figures of from 0.07 to 0.11 seconds. Additional data on the split second pulmonic sound and the third heart sound are presented.

Simultaneous phonocardiograms and fluorocardiograms in a normal subject and in a patient with mitral stenosis, both having an opening sound or snap of the mitral valve, allowed correlation of the ventricular fluorocardiogram with the sound tracing in regard to the isometric relaxation period.

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[10] Boone, B. R., Chamberlain, W. E., Gillick, F. G., Henny, G. C., and Oppenheimer, M. J., *Am. Heart J.*, 1947, **34**, 560.

16606

Staphylococcus aureus: Drug-fastness Studies with Penicillin and Sulfactin.*†

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(Introduced by A. N. Richards.)

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Sulfactin, a new antibiotic produced by an Actinomyces, was described by Junowicz-Kocholaty, Kocholaty, and Kelner.[1] Its bacterial spectrum, toxicity, and therapeutic action in mice were described by Morton.[2] It was found that there was a very favorable ratio between the toxic dose and the therapeutic dose in mice which had been infected with *Diplococcus pneumoniae*, type 1. Sulfactin is active against gram-positive microorganisms, primarily, and in this respect it resembles penicillin. We were interested to learn if a culture of a gram-positive microorganism such as *Staphylococcus aureus* readily acquired an increase in its resistance to sulfactin, whether a sulfactin-resistant strain of staphylococci showed any change in its susceptibility to penicillin, and whether a penicillin-resistant strain of staphylococci showed any increase in its resistance to sulfactin.

The supply of pure sulfactin was that mentioned previously.[2] The commercial sodium salt of penicillin was used. Stock solutions of both antibiotics were prepared in sterile distilled water and stored in the dry-ice box. Bacto-nutrient broth, adjusted to pH 7.4 was employed as the culture medium.

Penicillin-resistant and sulfactin-resistant staphylococcal cultures were developed *in vitro* by growing the test organism in the presence of varying concentrations of the antibiotics. From the stock solutions of each antibiotic serial two-fold dilutions were prepared in Bacto-nutrient broth, pH 7.4. All of the dilution tubes were then seeded with one drop of broth culture of *Staphylococcus aureus* and the tubes incubated at 37°C. Observations were usually made after 24 hours, but in some instances the incubation period was 48 or 72 hours. The tubes in the penicillin series and in the sulfactin series containing the highest concentration of the antibiotic which showed visible growth of staphylococci were selected for subculturing. New series of dilutions of penicillin and sulfactin were prepared and inoculated from the tubes in the previous series which had been selected for subculturing. This procedure was repeated until penicillin-resistant and sulfactin-resistant cultures of staphylococci were obtained. The extent of the tolerance of the cultures for the two antibiotics is shown in Table I. Parallel transfers of the parent culture of staphylococci were made in the Bacto-nutrient broth for a control.

Tests to determine the sensitivity to penicillin and to sulfactin were then made with the penicillin-resistant strain, the sulfactin-resistant strain and the normal culture of staphylococci. In becoming resistant to penicillin the staphylococcal culture did not change in its sensitivity towards sulfactin. Likewise when the culture of staphylococci became resistant to sulfactin it did not change in its sensitivity towards penicillin. Typical results of repeated tests are presented in Table II.

Results. After 7 serial transfers of a staphylococcal culture in Bacto-nutrient broth containing varying amounts of sulfactin, the culture grew in the presence of 10 μg of the drug. This represents an increase in resistance of the culture to the drug of over 1100 times that of the original strain (Table I). Under similar conditions the staphylococcal culture developed a resistance to penicillin of slightly over 32 fold. The acquisition of a tolerance for either penicillin or sulfactin by the staphylococcal culture was not accompanied by an increase in tolerance for the other antibiotic.

Discussion. The value of using drug-fast strains of microorganisms in studying new chemotherapeutic compounds was described by Ehrlich[3] and was advocated in the study of new antibiotics by Stansly,[4] Eisman *et*

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* This project has been supported by the Smith, Kline and French Laboratories, Philadelphia, Pa.

† This is based upon a thesis in medical bacteriology presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Master of Science.

‡ Present address: School of Tropical Medicine, Government of Puerto Rico, San Juan, Puerto Rico.

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[2] Morton, H. E., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 345.

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[4] Stansly, P. G., *Science*, 1946, **103**, 402.

TABLE I.

Development of Resistance *in Vitro* of a Strain of *Staphylococcus aureus* (P 210) to Sulfactin and Penicillin.

Antibotic	Sulfactin														Penicillin									
Tube No.	c	1	2	3	4	5	6	7	8	9	10	11	12	13	c	1	2	3	4	5	6	7	8	9
Approximate conc. of antibiotic per ml of medium	0	10 µg	5 µg	2.5 µg	1.25 µg	0.625 µg	0.312 µg	0.156 µg	0.078 µg	0.039 µg	0.019 µg	0.009 µg	0.004 µg	0.002 µg	0	10 U	5 U	2.5 U	1.25 U	0.625 U	0.312 U	0.156 U	0.078 U	0.039 U
Subculture 1	4	—	—	—	—	—	—	—	—	—	—	—	4	4	4	—	—	—	—	—	—	3	3	3
2	4	—	—	—	—	—	—	—	—	—	3	3	4	4	3	—	—	—	—	—	1	2	2	
3	3	—	—	—	—	—	—	—	—	2	3	3	3	3	4	—	—	—	—	—	1	2	2	
4	4	—	—	—	—	—	—	4	4	4	4				3	—	—	—	—	+	1	2	2	
5	4	—	—	—	—	—	4	4							4	—	—	2	2	3	3	4	4	
6	4	—	—	—	—	+	2	3							4	—	+	2	3	4				
7	4	3	3	3											4	+	2	3	4					

— = no growth.
4 = maximum amount of growth.
3 and 2 = proportionate amount of growth between 4 and 1.
1 = smallest amount of growth readily detected macroscopically.
+ = trace, or growth barely detectable macroscopically.

TABLE II.

Comparison of Typical Results Obtained by Testing the Parent Culture of *Staphylococcus aureus*, P 210, and the Penicillin-adapted and Sulfactin-adapted Strains of This Culture Against Penicillin and Sulfactin.

Antibotic	Sulfactin										Penicillin									
Tube No.	c	1	2	3	4	5	6	7	8	9	c	1	2	3	4	5	6	7	8	9
Concentration of antibiotic per ml of medium	0	10 µg	5 µg	2.5 µg	1.25 µg	0.625 µg	0.312 µg	0.156 µg	0.078 µg	0.039 µg	0	10 U	5 U	2.5 U	1.25 U	0.625 U	0.312 U	0.156 U	0.078 U	0.039 U
Staphylococcus aureus																				
Parent culture	4	—	—	—	—	—	—	—	3	4	4	—	—	—	—	—	—	1	2	2
Penicillin-adapted strain	4	—	—	—	—	—	—	—	3	4	4	+	2	3						
Sulfactin-adapted strain	4	4	4	4							4	—	—	—	—	—	—	1	2	3

—, 1, 2, 3, and 4 = amount of growth as described in footnote to Table I.

al.,[5] Graessle and Frost,[6] and Sullivan *et al.*[7]

Therapeutic failures, as a result of a particular strain of an infecting parasite acquiring or possessing a tolerance to the drug, have been known since the advent of chemotherapy. The superior therapeutic action effected by the use of more than one chemotherapeutic agent was pointed out by Laveran[8] in his work with trypan red and arsenous acid in experimentally induced trypanosomiases. The rational of a plan of drug rotation in the case of certain bacterial infections was pointed out by Feirer, Meader and Leonard[9] and again by Duemling and Horton.[10]

Ungar[11] first demonstrated the synergistic effect *in vitro* and *in vivo* of chemotherapeutic agents (para-aminobenzoic acid and sulfapyridine) on an antibiotic (penicillin) and Carpenter, Bahn, Ackerman, and Stokinger[12] pointed out the advantages of combining 3 chemical substances with penicillin in order to prevent the development *in vitro* of drug-fast strains of the gonococcus. Although sulfactin is active against the same types of microorganisms against which penicillin is active, it nevertheless has a possible role in the field of chemotherapy.

It will be noted that the sensitivity of the staphylococcal strain to sulfactin at the beginning of the studies (Table I) is slightly different from that found for the parent culture later in the studies (Table II). A possible factor in explanation of this may be that a different lot of stock solution was employed in the later tests but this is not a complete explanation.

The magnitude of the resistance to sulfactin developed by a strain of staphylococci was intermediate between the increase of the same strain of staphylococci to penicillin and the increase in resistance of a strain of *Escherichia coli* to streptomycin.[13]

Summary. A strain of staphylococci in becoming resistant to penicillin did not show an increase in its resistance to sulfactin. A similar strain of staphylococci in becoming resistant to sulfactin did not show an increase in its resistance to penicillin. The increase in resistance to sulfactin developed by the strain of staphylococci was intermediate between the increment in resistance developed by staphylococci to penicillin and the increase in resistance developed by a strain of *E. coli* to streptomycin.

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16607

Quantitative Chemical Analysis of the Adrenal Glands of Wild Norway Rats.

John Nichols. (Introduced by A. T. Miller, Jr.)

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Rogers and Richter[1] pointed out that the wild Norway Rat has an adrenal gland 2-3 times as large as that of the domestic albino Norway Rat. This difference in size is limited to the cortex. Since the cortical hor-

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[1] Rogers, P. V., and Richter, C. P., *Endocrinology*, 1948, **42**, 46.

TABLE I.

	Wt of animal in g	Total fat	Total cholesterol	Source
Wild Norway	250-475	6.5% ± 1.2*	3.7% ± 0.8*	This paper
Domestic				
Wistar	300	8.7	5.1	Nichols[2]
Sprague-Dawley	200		5.48	Tepperman[3]
Long-Evans	(Adult)		5.28	Sperry[4]
Sprague-Dawley	250		5.84	Sayers *et al.*[5]
Yale	24-day-old		3.03	Sayers *et al.*[6]
Long-Evans	284		3.45	Levine[7]

$$* = \pm \sqrt{\frac{\Sigma d^2}{N}}$$

mone is likely formed from the cholesterol present in the gland it would be interesting to compare the cholesterol concentration in the adrenals of the two strains of animals. Such data are not in the literature. Accordingly the following study was carried out.

The adrenal glands of 41 wild Norway Rats* were subjected to a chemical analysis which has been previously described.[2] These rats were trapped during a typhus survey program currently being carried out in this state. They varied in size from 250 g to 475 g and were about equally distributed between the two sexes. Pregnant females were not used.

Results. The results were tabulated in comparison with the findings of other investigators on the adrenal of the domestic rat.

Discussion. In their paper Rogers and Richter present an interesting discussion based largely on the fact that the lean, aggressive, wild rat has a "faster pace of living" than does the obese, sedentary, domestic rat. It is well known that the adrenal cortex is an important factor in stresses not far different from this (Selye[8]). The adrenal cortical hormone is a cyclopentophenanthrene compound closely related to cholesterol and the adrenal gland is a rich source of cholesterol. There is every reason to believe that the hormone is formed from the cholesterol present in the gland. (This is true of all glandular structures which arise from embryonic lateral plate mesoderm.) Sayers *et al.*[6] found that the adrenal cholesterol became depleted upon administration of adrenotropic hormone. Mason *et al.*[9] have reported an increased urinary output of keto-steroids in humans after administration of adrenotropic hormone. At least one biologically active compound can be formed from cholesterol; Bloch[10] fed cholesterol labeled with deuterium to pregnant women and recovered it as pregnandiol in the urine.

From the data presented here it is apparent that both total fat and cholesterol are present in lower concentration in the adrenals of wild rats than in domestic rats. Assuming the 5% figure to be the correct one for the cholesterol concentration in the adrenal of the domestic rat, the wild rat has approximately 66% as much cholesterol. However, since Rogers and Richter[1] have shown the cortex of the wild rat to be 2-3 times larger than the cortex of the domestic rat, it is apparent that the wild rat has much more cholesterol available for hormone production than does the domestic rat of comparable size. The writer is unable to offer any explanation for the disparity in the two sets of figures by different investigators shown in the table.

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* These rats were kindly made available by Drs. H. F. Schoof and G. C. Barden of the State Health Department, Raleigh, N. C.

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It should be pointed out that these rats were caught in spring steel traps and in some cases may have remained in the trap for as long as 12 hours. The emotional disturbances and trauma associated with this may have caused a lowering of the original cholesterol.

Summary. Quantitative analysis of the adrenal glands of the wild Norway rat reveals that the total fats comprise 6.5% of the gland and that of this 3.7% are cholesterols. These figures are lower than the values for the adrenal gland of the domestic rat. However, since the cortex of the adrenal gland of the wild rat is 2-3 times larger than that of the domestic rat, the wild rat has much more cholesterol available for hormone production.

16608

Complement Fixation in Human Sera Following Murine Typhus.

E. R. Rickard.

From the International Health Division, Rockefeller Foundation, and Florida State Board of Health.

Complement-fixing antibodies for murine typhus have been shown to persist in the sera of both human beings and rodents for long periods of time following infection.[1-3] The complement fixation test has been employed, therefore, to estimate the past incidence of typhus in human[4] and rodent[5] populations. For this reason, an appraisal of the accuracy of the test as an indicator of past infection should be of interest. During a recent state-wide survey of murine typhus in Florida,[6] clinical and epidemiological records were completed on 2055 persons who, during the years 1944, 1945 and 1946, had had typhus or had been strongly suspected of having had the disease. The present report will describe the results of complement fixation tests performed upon the sera of 404 persons whose cases were investigated during the survey.

Methods. All persons from whom serum specimens were obtained had had fever and constitutional symptons sufficiently severe to confine them to bed for a period of at least 10 days. Furthermore, all these persons had had relatively close contact with rats and their ectoparasites, and there was epidemiological evidence that they could have contracted typhus.

Sera were collected aseptically at intervals varying from 7 days to 3 years and 11 months following the onset of illness, and were stored at icebox temperature for periods varying from 7 days to 4 months before examination. Immediately before testing they were inactivated by exposure to a temperature of 56°C for 30 minutes.

Antigen* was prepared by the Cox method[7] from the yolk sacs of chick embryos infected

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* We are grateful to Dr. Herald R. Cox of the Lederle Laboratories for furnishing the antigens used in this study.

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with murine typhus rickettsiae of the Wilmington strain. Lipoids were extracted with benzol and ether, and the antigen was purified by sodium sulfate precipitation in order to eliminate or reduce non-specific reactions with syphilitic sera.[8]

Hemolysin was titrated in 0.25 ml amounts in twofold dilutions, with starting dilutions of 1:200 and 1:300 followed by the addition of 0.25 ml of 3% washed sheep cells and 0.5 ml of guinea pig complement diluted 1:30. The highest dilution of amboceptor which showed complete hemolysis after incubation for 30 minutes at 37°C constituted one unit. This dilution divided by 3 was employed in the tests. Complement was titrated in 0.5 ml amounts in the presence of 0.25 ml of diluted antigen and 0.25 ml of normal saline. The hemolytic system of 0.5 ml of 1.5% sensitized cells was added, and results were read after incubation for 30 minutes at 37°C. Two full units of complement in 0.5 ml amounts were used in the tests. Antigen was titrated in serial twofold dilutions of 0.25 ml amounts with serial twofold dilutions of the same amounts of a positive serum. Complement was added and, after 18 hours of icebox fixation, the above-mentioned hemolytic system was added. The titration was read after incubation for 30 minutes at 38°C. The highest dilution of antigen giving 4 plus fixation with the highest dilution of serum was considered the unit of antigen. Two units in 0.25 ml amounts were used in the tests.

Serum specimens were diluted in 0.25 ml amounts in original dilutions ranging from 1:2 to 1:128. Tests were carried out in the same manner as described under titration of antigen, with the exception that the highest serum dilution in which 3 plus or greater fixation was observed was considered the end point. In tests for each day, 2 known positives and 3 known negative control sera were included, as well as a control titration of the complement with the same amount of antigen as was used in the test. Anticomplementary controls were included for all sera in the lowest dilution, and anticomplementary sera were

TABLE I.

Correlation of Clinical and Weil-Felix Agglutination Findings During Illness with Titers Observed in Late Complement Fixation Tests.

Group No.	Findings at time of illness	No. sera examined	No. positive sera	% positive	Titers Complement fixation tests 0	1:2	1:4	1:8	1:16	1:32	1:64	1:128
I	Rash. Weil-Felix 1:320 or more	124	109	88	15	2	5	18	34	27	19	4
II	No rash. Weil-Felix 1:320 or more	79	67	85	12	1	6	13	14	22	7	4
III	Rash. No serological exam.	105	77	73	28	0	5	14	12	25	14	7
IV	Rash. Weil-Felix 1:160 only	21	14	67	7	0	1	3	4	5	1	0
V	No rash. Weil-Felix 1:160 only	16	8	50	8	0	0	2	3	3	0	0
VI	No rash. No serological exam.	59	38	65	21	2	3	2	15	10	4	2
	Total	404	313	77	91	5	20	52	82	92	45	17

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[8] Van der Scheer, J., Bohnel, E., and Cox, H. R., *J. Immunology*, 1947, **56**, 365.

TABLE II.
Titers of Positive Complement Fixation Tests in Relation to Time After Onset of Illness.

Months after onset of illness	No. positive sera	Titers							Mean positive titers
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	
12	29	0	2	3	2	11	5	6	1:34
12-23	67	0	1	6	17	24	12	7	1:30
24-35	123	4	6	28	31	38	16	0	1:18
36-47	94	1	11	15	32	19	12	4	1:18
Total	313	5	20	52	82	92	45	17	1:21

excluded from the series.

Results. In Table I, the persons on whose sera complement fixation tests were performed have been classified in groups according to the history of the presence or absence of a typical typhus rash and the presence of Weil-Felix agglutination in significantly positive or suggestive titer at the time of their illnesses. It would seem highly probable that the persons in Groups I and II had suffered from murine typhus. Weil-Felix agglutination titers of 1:320 or more are generally considered as diagnostic of rickettsial infection. Rocky Mountain spotted fever was the only rickettsial disease known to occur in Florida which might have caused the symptoms of, and the findings on, the patients in these two groups. In no instance did epidemiological evidence suggest this disease. Moreover, of the 91 sera negative for typhus, 66 were examined by the same complement fixation test for Rocky Mountain spotted fever. With the exception of 13 sera in which positive results were obtained in 1:2 dilution only, all of the sera examined for Rocky Mountain spotted fever were negative. Because of the lack of both clinical and epidemiological evidence of Rocky Mountain spotted fever, these positive results in low titer were considered non-specific.

From the data on Groups I and II presented in Table I, it would appear that the complement fixation test was approximately 85 to 88% accurate as an indicator of past infection with murine typhus for periods up to 3 or 4 years following illness. It was also apparent that positive titers were quite high. The most common titer was 1:32, and the mean was 1:21. In the individuals included in Groups III to VI, the diagnosis of typhus was not considered completely established in every case. The possible inclusion of diseases other than typhus in these groups might account for the lower percentage of positive sera observed in the groups. This fact appeared to have no influence on the mean positive titers for Groups I and II as compared with Groups III to VI with titers of 1:21 and 1:22 respectively.

In Table II, persons with positive titers have been classified in one-year periods according to the time elapsed between illness and the taking of serum for complement fixation. The diminution in mean positive titers observed in the yearly intervals after infection was not statistically significant. This observation suggested that no pronounced loss of positive titer had occurred with the passage of time.

As a control upon the specificity of the test, the sera of 20 inmates of a New York State correctional institution were examined for murine typhus by the same technic. Of these sera, 17 were negative and 3 fixed complement in 1:2 dilution, one serum with 4 plus fixation and 2 with 3 plus. None of the 3 subjects had positive serological reaction for syphilis. Two had never been out of New York State and had never taken typhus vaccine. One, however, had had 2 years of naval service on the island of Guam ending a few months before the specimen was taken. It was, therefore, possible that this person had received typhus vaccine or may have been infected during the course of his travels. However, there had been no history of illness during the period of his absence from New York State. It would appear that at least 2, and probably all 3, of these positive reactions were nonspecific. In consideration of the higher titers commonly observed in persons who had had typhus, the occasional occurrence of non-specific reaction in low dilution of serum would not seem to detract greatly from the utility of the test.

Summary. Complement fixation tests for murine typhus were performed upon the sera of 203 persons who, it is highly probable, had suffered an attack of murine typhus 7 days to 3 years and 11 months previously. The tests were positive in 85 to 88% of the cases, and in most instances the positive titers were high. Among 201 persons suspected of having had typhus, though the diagnosis was not conclusively established, the per cent of positive reactions was less, but the titers of positive sera did not differ significantly from those of the group in which the diagnosis was considered to be established. Up to periods of 4 years after infection, mean positive titers at yearly intervals did not suggest a pronounced loss of titer with passage of time. With the exception of infrequent reactions at 1:2 dilutions in the sera of persons who had not had typhus, the test was found to be specific.

16609

Studies on Phosphorus-Containing Compounds in Normal and Polioencephalitic Mouse Brain.*

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(Introduced by M. B. Visscher.)

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Chemical investigations in the field of neurophysiology have shown that all conditions leading to a depressed nerve function are associated with a breakdown of phosphocreatine.[1] In nerve cells in which chromatolysis has been produced by axon section a markedly increased acid phosphatase activity has been found. This increased activity was proportional to the degree of chromatolysis and was probably associated with increased nucleoprotein synthesis or degradation.[2]

In regenerating neurons which are resistant to the infection with the poliomyelitis virus a decrease of phosphocreatine could also be shown. This change together with the reduction of cytochrome oxidase and succinic dehydrogenase activity in regenerating neurons resembled the effect of cyanide on brain metabolism.[3] Changes in the acid soluble phosphorus compounds in the brain in poliomyelitis have been described, consisting in a greatly increased content of adenosine-triphosphate and a markedly decreased content of phosphocreatine and residual organic phosphate.[4]

Experiments. In the present studies phosphorus-containing compounds were determined in normal mouse brain tissue and compared with those found in the brain of animals infected with polioencephalitis. Five weeks old mice of the C_3H strain received from the colony of Dr. John J. Bittner, University of Minnesota, were inoculated intraperitoneally with the MM poliomyelitis virus. At the height of the infection the animals were decapitated, the brain rapidly removed and homogenized in cold 5% trichloracetic acid. Phosphorus compounds were determined by the method of Schneider[5] which allows for

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

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[4] Kabat, H., *Science*, 1944, **99**, 63.

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[illegible]

Avg. normal [illegible]

Avg. infected [illegible]

P [illegible]

[illegible]

The separation of phosphorus compounds was [illegible]

[illegible]

Table I shows the values of phosphorus-containing compounds in normal and infected brain tissue.

Conclusions.— Determinations of phosphorus-containing compounds of the whole brain tissue taken from normal mice and from mice suffering from polio-encephalitis produced by the [illegible] poliomyelitis virus did not reveal any significant differences [illegible]

16610

Fertility in Rabbit as Affected by the Dilution of Semen and the Number of Spermatozoa.*

PEILIEU CHENG AND L. E. CASIDA.

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Walton[1] first demonstrated in the rabbit that fertility is influenced by the number of spermatozoa introduced into the vagina; maximum fertility was obtained when the number of spermatozoa was more than 1,000,000. The semen was diluted in 0.9% sodium chloride solution and 3 cc of semen suspension was used per insemination. Rowlands[2] used Baker's solution to dilute the semen and 2 cc of such semen suspension was deposited per insemination. He found the same number of spermatozoa necessary for maximum fertility as Walton did and sterility was reached when the number was dropped to 100,000. Chang[3] also used 0.9% sodium chloride solution as the diluent of rabbit semen with only 1 cc per insemination, and obtained a much lower figure on the number of spermatozoa for both maximum and minimum fertility (for maximum fertility: 330,000-420,000; for minimum fertility: 40,000). Chang[4] also tested the effect of dilution on the fertilizing capacity of rabbit sperm by inseminating the same number of spermatozoa suspended in various volumes of physiological saline (1.0 cc, 0.4 cc and 0.1 cc). The advantage appeared to be with the least volume.

The present studies attempt to determine the minimal number of sperm for fertility from insemination into the vagina and into the uterine horns with highly diluted semen. Further, an attempt was made to determine the effects of high dilution with physiological saline and Krebs solutions upon the motility of spermatozoa. Also the effect of high dilution with Krebs, and its consequent effect upon motility, upon the fertilizing capacity of the sperm was determined.

Experiment I. Fertility in relation to number of spermatozoa.

Material and methods. Thirty-six female rabbits were used in this study. All animals were in heat and mated to vasectomized bucks to induce ovulation. Semen samples were collected from 2 or 3 normal males with an artificial vagina[5] and pooled together. The number of spermatozoa was determined from the average counts of 2 independent hemocytometer samples. Four hundred hemocytometer squares were counted in each case and no pairs of counts were accepted which showed differences of statistical significance between them. The initial motility of spermatozoa was estimated in ranks or grades. The semen was then diluted with Krebs solution, modified according to Lardy and Phillips[6] until the desired number of spermatozoa per unit of volume was obtained and then kept in a warm water bath at approximately 37°C for the short time until it was used.

Four females were used in each trial with 2 in each group. In the "vaginal group" 0.2 cc of semen suspension was deposited into the anterior portion of the vagina by an inseminating tube; in the "uterine group," a laparotomy was made under light anesthesia and 0.1 cc of the same semen suspension was injected into the tubal ends of each of the uterine horns with a ¼ cc tuberculin hypodermic syringe. Animals in both groups,

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TABLE I.
Fertility in Relation to Number of Spermatozoa.

No. sperm. per insem. × 1000	Dilution rate	Vaginal group			Uterine group		
		No. eggs	% fert.	Avg % fert.	No. eggs	% fert.	Avg % fert.
92	1/1000	10	100	100	8	100	100
		7	100		0	—	
61	1/1000	7	71	36	5	0	0
		5	0		6	0	
46	1/1000	11	100	100	7	100	100
		8	100		0	—	
32	1/2500	8	50	50	10	70	35
		0	—		10	0	
24	1/2000	13	100	81	12	100	100
		8	62		5	100	
17	1/3500	9	0	0	5	0	0
		9	0		9	0	
17	1/4500	9	0	0	8	0	14
		16	0		11	27	
16	1/3500	9	11	20	12	0	15
		10	30		10	30	
16	1/5000	8	62	62	6	0	0
		0	—		7	0	
Group fertility (unweighted avg)				50			40

therefore, received approximately equal amounts of suspension containing approximately equal numbers of spermatozoa.

The insemination was made about 1½ to 3½ hours after the sterile mating (only sperm free seminal fluid deposited in vagina by the vasectomized male). Autopsy was made 25 to 30 hours after insemination; the oviducts were flushed, and fertilization of the ova was determined microscopically from the presence of 2 to 8 equal blastomeres.

Results. The findings of the present study show that partial fertility can result from a very small number of spermatozoa per insemination, as few as 16,000 (Table I). This was true in both vaginal and uterine inseminations. Maximum fertility was obtained with a number of spermatozoa around 90,000, and fertility was affected greatly when the number was cut below 20,000. A negative relationship was found between the dilution rates and the fertilizing capacity of the spermatozoa but the effect of a small number of sperm was confounded with the effect of high dilution. The effects of dilution rate upon motility were observed incidentally but the effect of this factor upon fertility also was confounded with number and concentration. The average fertility resulting from vaginal insemination was higher than that from uterine insemination (50% vs 40%). However, statistical analysis of the data from the 2 groups failed to show a significant difference between them ("t" test: $P > .05$). If the vaginal group could have been inseminated at laparotomy similar to the uterine group, the data could be considered more critical in comparing the effects of the two placements upon fertility.

Experiment II. Motility of spermatozoa as affected by high dilutions with different diluents. The above data show that the number of spermatozoa necessary either for maximum or for partial fertility is less than that previously reported. The discrepancy between different results may be due to the difference in diluents. The effects of high dilution upon motility of spermatozoa in a series of dilutions with both 0.9% sodium chloride and Krebs solutions were investigated next.

Material and methods. Twelve semen samples from 9 males were collected at different times and diluted in 8 successive dilutions with both 0.9% sodium chloride and Krebs solutions from 1:100 to 1:12800. Semen was introduced into containers of adequate sizes by a 0.01 cc graduated pipette; the diluent was then added slowly, and shaken gently in order to obtain an even dispersion

TABLE II.
Motility and Highest Dilution Showing Motility of Rabbit Spermatozoa in Two Diluents.

	Avg % motility in 8 dilutions (from 1:100 to 1:12800)		Highest dilution showing motility	
Sample No.	0.9% NaCl	Krebs	0.9% NaCl	Krebs
1	29.4	36.2	3200	12800
2	21.2	36.2	800	3200
3	13.1	24.1	400	12800
4	6.4	20.6	400	6400
5	19.4	32.0	800	6400
6	6.3	10.4	200	3200
7	13.4	20.6	800	3200
8	1.4	5.1	200	400
9	30.1	38.7	3200	6400
10	8.1	13.9	400	1600
11	23.1	43.9	1600	3200
12	0.9	6.9	400	800
Avg	14.4	24.1	1033	5033

of spermatozoa. All semen suspensions were kept in the warm water bath for 30 minutes and the percentage of spermatozoa showing progressive motion then estimated.

Results. Almost without exception, the average motility in the 8 dilutions and also the highest dilution rate supporting motility was higher for Krebs solution than for the 0.9% sodium chloride solution (Table II), and a trend was shown in both diluents that the higher the dilution rate the lower the motility (Table III). Statistical analyses showed that the motility of spermatozoa varied significantly from one dilution to another, and the average difference between the diluters was also highly significant ("F" test, $P<0.01$).

TABLE III.
Progressive Motility of Rabbit Spermatozoa in Dilutions from 1:100 to 1:12800.

	Avg percentage motility in	
Dilution	0.9% NaCl	Krebs
1: 100	48.3	57.9
1: 200	37.7	47.5
1: 400	18.2	35.5
1: 800	8.0	23.8
1: 1600	2.5	17.4
1: 3200	0.5	8.6
1: 6400	0	1.2
1:12800	0	0.6
Avg	14.4	24.1

Experiment III. Fertility as affected by rates of dilution and motility of spermatozoa. The low motility observed in the high dilutions causes one to recall the results in Experiment I, in which the rates of dilution were from 1/1000 up to 1/5000. Considering the fact that the average highest dilution showing motility was 1:1033 in 0.9% sodium chloride solution and 1:5033 in Krebs solution, it was suspected that the motility of the spermatozoa is one of the determining factors in fertilization and the difference in solutions accounts for the discrepancy between present results and those of former workers. The effect of dilution upon the fertilizing capacity of the spermatozoa was therefore studied next.

Material and methods. The same small number of spermatozoa, viz., 20,000 all from the same sample of semen, were suspended in 0.1 cc, 0.2 cc, 0.4 cc and 0.8 cc of Krebs solution. Four animals were used for each trial, each animal received a different volume of suspension; altogether there were 7 trials in this study. The same procedure as mentioned in Experiment I was followed except the semen suspension was deposited in the vagina in all animals. The motility of spermatozoa was estimated on each semen suspension before the insemination.

Results. The fertility data are based on 22 animals; the remaining 6 failed to ovulate. The average fertility in each of the 4 different volume groups was: 0.1 cc, 60.0%; 0.2 cc, 60.5%; 0.4 cc, 37.2%, and 0.8 cc, 38.2% (Table IV) for a range of 23.3%. The

TABLE IV.
Fertility of Rabbits in Relation to Dilution and Motility of Spermatozoa.

Motility rank*	200,000/cc 0.1 cc/insem.		100,000/cc 0.2 cc/insem.		50,000/cc 0.4 cc/insem.		25,000/cc 0.8 cc/insem.		Total No. eggs	Avg % fertility
	No. eggs	% fert.	No. eggs	% fert.	No. eggs	% fert.	No. eggs	% fert.		
1					6	0	8	0		
							11	91		
							7	0	32	22.7
2					8	50	10	100		
					7	0			25	50.0
3			7	100	6	100	5	0		
			8	25						
			9	0					35	45.0
4	9	100	8	38	11	73				
	11	0			9	0			48	42.2
5	7	100	7	100						
	9	0	8	100						
	8	100							39	80.0
Total No. eggs	44		47		47		41		179	
Avg % fertility		60.0		60.5		37.2		38.2		

* 1, no motion at all; 2, oscillatory motion; 3, motility from 1% to 25%; 4, motility from 25% to 50%; and 5, motility from 50% to 75%.

different motility groups, however, showed a range of 57.3% from 22.7 for grade 1 motility up to 80.0 for grade 5.

A significant correlation was found between motility and fertility, 0.43, but an insignificant correlation was shown between sperm concentration and fertility, 0.22. The motility of the spermatozoa and the sperm concentration showed a rather high correlation, 0.78. The partial correlation between fertility and motility with concentration held constant was 0.42 whereas between fertility and concentration with motility held constant it was —0.17 and insignificant. Under the conditions of high dilutions, the motility of the spermatozoa appears to have some value for predicting fertility. It also appears that much of the deleterious effect of high dilution upon fertility of sperm is brought about through its inhibitory action upon motility.

Summary. Female rabbits were inseminated with semen diluted with Krebs solution at rates of 1/1000 up to 1/5000 and 0.2 cc of suspension were used. The number of spermatozoa effective for partial fertility was 16,000, and for maximum fertility was around 90,000. The group percentage of fertility resulting from vaginal insemination was slightly higher than from uterine insemination (50% vs 40%); this difference was not of statistical significance.

When semen samples were diluted from 1:100 up to 1:12800, the motility of the spermatozoa decreased significantly as the rates of dilution increased and the average motility shown in Krebs solution and the maximal dilution that would support motility was significantly higher than in 0.9% sodium chloride solution.

When females were inseminated with a small number of spermatozoa (20,000) suspended in different volumes of Krebs solution, the average percentages of fertility were higher in the smaller volumes. The motility of the spermatozoa, however, appeared to have a greater effect on the fertility than the dilution.

16611

Some Microbiological Properties of Synthetic Nucleosides.*

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Increasing interest in the roles of pyrimidines, purines, nucleosides, and other nucleic acid derivatives in the living cell has directed the attention of many investigators to various biological systems which utilize these compounds. Through X-ray irradiation, Beadle and co-workers[1] obtained mutants of *Neurospora crassa* which required added uracil or uridine for growth. Likewise, mutants of *Escherichia coli* which require uracil for normal growth have been developed and studied.[2]

The *L. casei* factor (pteroylglutamic acid) requirements for the growth of *Lactobacillus casei, Streptococcus faecalis R,* and certain other microorganisms can be fulfilled by thymine.[3]

Because specific metabolite antagonists have been useful in determining some of the biological functions of metabolites as well as changing the growth characteristics of living cells, we synthesized a number of derivatives of uracil[4] and thymine[5] as possible antagonists of these metabolites. The possibility that these derivatives might enhance the activity of the corresponding natural metabolite was equally interesting.

Emerson and co-workers[6] found that a specific antagonist of riboflavin was obtained when the ribityl group was replaced by a dulcityl group. This suggested the possibility of producing an antagonist of uridine by substituting galactose or other sugars for ribose of the nucleoside molecule.

Kuhn and co-workers[7] reported the 6,7-dichloro derivative of riboflavin as a specific antagonist of riboflavin. Hitchings, Falco and Sherwood[8] found that 5-chlorouracil and 5-bromouracil were antagonists of thymine. In both of these instances the substitution of a chlorine atom for a methyl group produced an antagonist. Since Loring and Pierce[9] found natural uridine from 10 to 60 times more effective than uracil as a growth promotor for a uracil-less mutant of *Neurospora*, it seemed possible that the ribosyl and other glycosyl derivatives of 5-chloro- and 5-bromouracil might be more effective thymine antagonists than 5-chlorouracil or 5-bromouracil.

To test these various possibilities, the ribosyl, arabinosyl, glucosyl, and galactosyl nucleosides of uracil[4] and thymine[5] were synthesized, as well as the corresponding 5-chloro- and 5-bromouracil derivatives. Although synthetic 1-D-ribosyluracil is chemically similar to naturally occurring uridine it has different physical properties.[10] Recent work of Todd and co-workers[11] suggests that,

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although natural and synthetic nucleosides are beta-glycosides, the sugars differ in their ring structures. For these reasons the synthetic nucleosides might be expected to show microbiological properties different from uridine and from the uracil and thymine derivatives.

Results obtained in testing these synthetic nucleosides as possible metabolite antagonists for various microorganisms are reported in this paper.

Tests with Escherichia coli. Two strains of *E. coli* were used: a uracil-requiring mutant strain (550-460)‡ and a strain (American Type Culture Collection No. 9723) which synthesizes its own uracil. Good growth of the uracil mutant was obtained when uracil (0.005 to 0.04 mg per 7.5 ml) or natural uridine§ (0.01 to 0.1 mg per 7.5 ml) were added to the medium described by MacLeod.[12] The tests with this mutant were carried out in the presence of 0.015 or 0.02 mg of uracil per 7.5 ml of medium. These levels of uracil permitted about 50% of normal growth. When the uracil and thymine derivatives were tested in concentrations up to 1 mg per 7.5 ml of medium, only 5-chlorouracil was slightly stimulatory, while none of the following showed any growth-promoting or growth-inhibiting properties: 1-D-ribosyluracil, 1-D-arabinosyluracil, 1-D-glucosyluracil, 1-D-galactosyluracil, 1-D-ribosyl-5-chlorouracil, 1-D-arabinosyl-5-chlorouracil, 1-D-glucosyl-5-chlorouracil, 1-D-galactosyl-5-chlorouracil, 1-D-ribosyl-5-bromouracil, 1-D-arabinosyl - 5 - bromouracil, 1-D-glucosyl-5-bromouracil, 1-D-galactosyl-5-bromouracilthymine, 1-D-ribosylthymine, 1-D-arabinosylthymine, 1-D glucosylthymine, and 1-D-galactosylthymine. None of these compounds, when tested in concentrations up to 20 mg per 7.5 ml of medium, inhibited the growth of the American Type Culture Collection No. 9723 strain of *E. coli.*

Tests with Neurospora crassa.‖ The wild strain of *Neurospora crassa, Abbott 4A* was grown in the medium described by Horowitz and Beadle.[13] With the uracil-less mutant, *Neurospora crassa, 1298,* a typical growth curve was obtained when either uracil or natural uridine[9] was added to the medium. To obtain high enough concentrations without using too much of the various compounds, all tests with *Neurospora* were carried out in 10 ml of medium. In this amount of medium maximum growth was equivalent to 20 to 30 mg of dried mycelia. All tests with the uracil-less mutant were carried out in the presence of 0.1 mg uridine in 10 ml of medium. Of all the compounds listed in the previous section, when tested in concentrations up to 0.8 mg per 10 ml of medium, only 5-chlorouracil slightly stimulated the growth of the uracil-less mutant. None of the compounds in concentrations up to 20 mg per 10 ml inhibited the growth of either the wild or uracil-less strains of *Neurospora.*

Tests with Lactobacillus casei. All tests with *L. casei* (American Type Culture Collection, No. 7469) were carried out in the medium and according to the procedure described by Luckey, Briggs, and Elvehjem.[14] In the absence of added pteroylglutamic acid the growth reached a level of 20 turbidity units, whereas the addition of 0.010 mg of thymine permitted maximum growth equivalent to 130 turbidity units. The following compounds consistently stimulated the growth of *L. casei*: (The percentage values given in the parentheses indicate the relative molar activity as compared with thymine taken as 100%): 1-D-ribosylthymine (1.0%), 1-D-glucosylthymine (1.4%), 1-D-galactosylthymine (0.5%), and 1-D-arabinosyl-5-chlorouracil (0.3%). In the presence of 0.002 mg of thymine per 10 ml medium the following compounds were slightly stimulatory, whereas in the absence of thymine they were inactive: 1-D-glucosyl-5-chlorouracil, 1-D-galactosyl-5-chlorouracil, 1-D-arabinosyl-5-bromouracil, and 1-D-glucosyl-5-bromouracil. The following compounds were completely inactive when tested up to concentrations of 1.0 mg per 10 ml of medium: 1-D-arabinosylthymine, uracil, 5-chlorouracil, natural uridine, 1-D-ribosyluracil, 1-D-arabinosyluracil, 1-D-glucosyluracil, 1-D-galactosyluracil, 1-D-ribosyl-5-chlorouracil, I-D-ribosyl-5-bromouracil, and 1-D-galactosyl-5-bromouracil. At concentrations of 1.0 mg per 10 ml of medium none of the compounds showed any growth inhibitory properties, except uracil and 5-chlorouracil[8] which inhibited the growth in the presence of 0.002 mg of thymine. At concentrations of 20 mg per 10 ml of medium some of these synthetic nucleosides were slightly inhibitory.

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‡ The cultures of *E. coli,* American Type Culture Collection No. 9723 and the uracil mutant, *E. coli,* (550-460), were obtained through the courtesy of Dr. J. O. Lampen of the American Cyanamid Company of Stamford, Conn.

§ The authors are indebted to Dr. Hubert Loring of Stanford University for a generous sample of natural uridine.

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‖ The authors wish to thank Dr. Herschel Mitchell and Dr. Hubert Loring for kindly supplying cultures of *Neurospora.*

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Tests with Streptococcus faecalis R. The tests with *Strep. faecalis R*¶ (American Type Culture Collection, No. 8043) were made in the medium and according to the procedure referred to above.[14] The results obtained were almost identical with those obtained with *L. casei*. The compounds which showed some stimulation in the absence of thymine were: 1-D-ribosylthymine (2.2%), 1-D-galactosylthymine (0.7%), and 1-D-glucosylthymine (1.1%). The following compounds were slightly stimulatory in the presence of 0.002 mg of thymine per 10 ml of medium but were inactive alone: 1-D-ribosyl-5-chlorouracil, 1-D-arabinosyl-5-chlorouracil, 1-D-glucosyl-5-chlorouracil, 1-D-galactosyl-5-chlorouracil, 1-D-ribosyl-5-bromouracil, 1-D-arabinosyl-5-bromouracil, 1-D-glucosyl-5-bromouracil, and 1-D-galactosyl-5-bromouracil. None of the other compounds showed any growth-promoting activity.

At a concentration of 0.020 mg per 10 ml of medium 5-chlorouracil inhibited the growth to 100% in the presence of 0.002 mg of thymine. None of the other compounds up to a concentration of 1.0 mg per 10 ml showed any growth-inhibitory properties. However, when the concentration of these compounds was greatly increased, some of them were toxic. Galactosyluracil and 5-chlorogalactosyluracil, at levels of 10 and 5 mg, respectively, inhibited the growth to 50% of that obtained with 0.002 mg of thymine.

Summary. A number of synthetic nucleosides, and chloro- and bromo- derivatives of these nucleosides, were tested for growth-promoting and growth-inhibiting properties on *Escherichia coli,* strain 9723, and its uracil-requiring mutant, *E. coli,* strain 550-460; *Neurospora crassa,* wild strain Abbott 4A, and a uracil-less strain of *Neurospora crassa,* No. 1298; and on the growth of *Lactobacillus casei* and *Streptococcus faecalis R.*

The compounds tested were the 1-D-ribosyl-, 1-D-arabinosyl-, 1-D-glucosyl-, and 1-D-galactosyl- nucleosides of uracil, thymine, 5-chlorouracil and of 5-bromouracil. Of these 16 compounds, only a few at high concentrations showed inhibitory properties on the growth of the microorganisms tested. Some showed slight, but insignificant, growth-stimulatory activity.

The authors wish to show their appreciation to Miss Ann Coblenz, Miss Janet Tozer, Miss Evelyn Wyatt, and Mrs. Charmion McMillan for carrying out some of the microbiological tests.

¶ The authors wish to thank Dr. C. A. Elvehjem for a culture of *Strep. faecalis R.*

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Effects of Dehydrating Agents on Phosphatases in the Lymphatic Nodules of the Rabbit Appendix.

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In the course of a study on the quantitative correlation between phosphatase activities and histological changes in lymphatic tissue it has been necessary to determine storage properties and stability to dehydration of these enzymes in our material. The rabbit appendix was selected because it presents an almost diagrammatically homogeneous distribution of lymphatic nodules over a large area. This arrangement is ideal for histochemical examination of the effects of experimental modifications.[1] Rabbits were killed with nembutal and the appendix promptly removed, washed with saline, drained, weighed and dehydrated as indicated or minced and homogenized fresh. Strips weighing about one gram each were made up to 20 ml of homogenate. Determinations were carried out in triplicate at 30°C and 40°C with 2.5-5.0 mg of fresh weight of tissue in suspension. Disodium phenylphosphate (0.5%) was used as substrate with barbital or glycine buffers. The reaction was stopped at the end of 30 minutes by addition of Folin-Ciocalteau phenol reagent. No activation was found with added magnesium. Tissues to be dehydrated were dropped into 50 volumes of absolute acetone, 80% acetone, absolute alcohol, 80% alcohol, absolute methyl alcohol (all at room temperature) or into isopentane chilled in liquid nitrogen. The samples frozen in isopentane were dehydrated at –40°C *in vacuo*. The other samples were left in the fixative at 5°C for 24 hours and then drained and homogenized in water in a Potter-Elvehjem homogenizer.

Alkaline phosphatase. The optimum for alkaline phosphatase activity was found to be at pH 9.8 with 20% less activity at 9.6 and 10.1. Freshly homogenized appendix gave values for 6 different rabbits of 7.0 to 11.0 μg phenol liberated per milligram fresh weight of tissue in 30 minutes at 30°C. The activity at 40°C was 1.35 times as great. Single values in the triplicate determinations at each step agreed within 1%. Taking the fresh homogenate as 100% activity we found in the different specimens 95 ± 5% activity after storage of the homogenate at 5° C for 24, 48, 72 and 96 hours. The frozen dried tissue gave 85 ± 8% of the fresh value at periods of 1, 48 and 96 hours after homogenizing. After fixation in absolute methyl alcohol only 15% activity remained. When fixed for 24 hours in acetone or alcohol and then homogenized, the immediate values were as follows: absolute acetone, 80% acetone and absolute alcohol gave 75 ± 5% of the fresh activity: 80% alcohol gave 65 ± 5% activity. The acetone or alcohol fixed enzyme preparations underwent further inactivation upon storage as homogenates at 5°C at comparable steady rates so that 72 hours later about two-thirds of the initial values after fixation were obtained. Each of these fixed samples carries about 3% of the fixative into the homogenate suspension. In this material 80% alcohol has always preserved less alkaline phosphatase activity than either absolute alcohol or acetone which is at variance with the results of Stafford and Atkinson.[2] The frozen dried material has only slightly higher activity than the absolute alcohol and acetone fixed material but it is as stable upon storage as are fresh tissue homogenates. Frozen dried material stored dry in a desiccator at 25°C for 3 days showed a decrease of activity to about 30% but retained full activity for two weeks when stored at –20°C.

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* This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

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Acid phosphatase. Maximal acid phosphatase activity was obtained at pH 5.6 - 5.8 with about 10% less activity at 5.4 and 6.2. The freshly homogenized tissues gave individual values for the same 6 rabbits ranging from 10 to 14 μg phenol liberated per mg fresh weight of tissue in 30 minutes at 30°C. Taking the value one hour after homogenizing as 100% activity we found upon storage at 5°C: 75% activity after 24 hours, 70% after 48 hours, and 55% after 96 hours. Acetone (80% or 100%) preserved 25% of the fresh activity and upon storage of the homogenate for 72 hours this decreased to 15%. Alcohol (80% or 100%) preserved 15% of the fresh activity and upon storage as homogenate this declined rapidly (24 hours) to 2%. Methyl alcohol left negligible activity. The frozen dried tissue gave 85% of the fresh activity and after 48 hours had 70% of the initial fresh value which was the same as that of the fresh material after storage for the same period. Frozen dried material stored dry in a desiccator at 25°C for 3 days showed a decrease of activity to about 15% but was stable at –20°C.

Effect of temperature of fixation. In a subsequent series of experiments with 4 rabbits it was found that for acetone fixed material distinctly better cytological fixation and better preservation of both alkaline and acid phosphatase was obtained when tissues were dropped into acetone at –20°C and stored at this temperature for 48 hours.

Effect of embedding in paraffin. A sample of acetone fixed material with an activity of 9.3 μg phenol per mg fresh weight at pH 5.6 and 8.4 μg phenol per mg fresh weight at pH 9.8 was transferred to benzene and then to paraffin at 60°C for 1 hour. A paraffin block was made and stored 24 hours at 5°C. Most of the paraffin was cut away and the small block extracted with carbon disulfide at 40°C in a Soxhlet apparatus. When homogenized in water this material showed 60% of the acid phosphatase activity of the original acetone fixed material and 55% of the alkaline phosphatase activity. Analyses of sections in which the weight of the section was estimated from its calculated volume is consistent with these findings. In the work on sections it was found that the phosphatases were stable in the paraffin block even at room temperature for several days but that sections in paraffin ribbons deteriorated at room temperature although they were stable for a week at –20°C.

Summary and Conclusions. Alkaline phosphatase in the rabbit appendix is almost as well preserved by fixation in chilled acetone as by the freezing drying technic. Acid phosphatase on the other hand is distinctly better preserved by dehydration in the frozen state than after acetone fixation. Acid phosphatase activities are maximal when determined promptly on fresh homogenates. Frozen tissues dehydrated in the frozen state and stored dry at –20°C gave acid phosphatase activities equal to 85% of fresh values and for homogenates stored at 5°C for 24-96 hours the values of fresh and frozen preparations were identical. Acetone and alcohol fixation gave marked reduction in acid phosphatase activity. Alkaline phosphatase activity of fresh tissue is reduced to about 75% of the fresh value by treatment with acetone or absolute alcohol at room temperature. The effects of temperature of fixation, storage in the dry state and embedding in paraffin are noted.

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Problems in Determining the Bacterial Flora of the Pharynx.*

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The role of bacteria in the pathogenesis of the common respiratory diseases has not been clearly defined. Attempts have been made to correlate the pharyngeal flora with the presence or absence of upper respiratory infection and to determine whether or not changes in flora occur during the course of illness.[1–14] The results have been inconstant and difficult to interpret. Consideration of the methods employed suggests that technical and nutritional limitations may have been responsible, in some instances, for apparent differences in flora, while in other instances, such limitations may have obscured changes in flora that were present.[12] A study has therefore been made to define some of the problems of interpreting the results of pharyngeal cultures and to develop a technic that would yield reproducible as well as interpretable data. The investigations were carried out in conjunction with other studies on respiratory disease.[15–18]

This report presents certain of the results under 3 headings: a) technic, b) quantitative growth and nutritional interdependence, and c) use of a selective medium for detecting *beta*-hemolytic streptococci.

Technic. Two of the technical difficulties

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† Members and professional associates of the Commission on Acute Respiratory Diseases were: John H. Dingle, Director; Theodore J. Abernethy; George F. Badger; Joseph W. Beard; Norman L. Cressy; A. E. Feller; Irving Gordon; Alexander D. Langmuir; Charles H. Rammelkamp, Jr.; Elias Strauss; Hugh Tatlock.

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[16] Commission on Acute Respiratory Diseases, *Am. J. Pub. Health*, 1945, **35**, 675.

[17] Commission on Acute Respiratory Diseases, *Johns Hopkins Hosp. Bull.*, 1946, **79**, 97.

[18] Commission on Acute Respiratory Diseases, *Medicine*, 1947, **26**, 465.

TABLE I.
Comparison of Results with Two Methods* of Examining Throat Swabs.

Organism	Dry swab positive Suspension positive	Dry swab positive Suspension negative	Dry swab negative Suspension positive	Dry swab negative Suspension negative
	No. of swabs			
Beta-hemolytic streptococci	2	1	4	69
S. aureus	1	0	3	72
H. influenzae	51	0	11	14
H. hemolyticus	52	3	4	17
Total	106	4	22	172

* See text.

in culturing the throat reside in (*1*) the transference of bacteria from the cotton swab to the culture plate in a manner such that the resultant growth will reflect quantitatively the various species of bacteria on the swab, and (*2*) the visual identification of species by their colonial morphology prior to picking the colony and identifying the pure culture so obtained.

The problem of transferring bacteria from the swab to the medium was approached by comparing the results obtained by steaking the swab directly on the medium in the manner previously employed,[12] with those obtained by suspending the material on the swab in broth prior to inoculation of the medium.

Each of 76 swabs, rubbed thoroughly over the tonsils and oropharynx, was streaked directly onto a blood agar plate. Each swab was then immersed in 5 ml of tryptose-phosphate broth. With the swab in place, the broth was drawn into and expelled from a 5 ml pipette 20 times. From this tube 0.5 ml was then transferred to a second 5 ml portion of broth, and mixed as above with the same pipette. Using a 1.0 ml pipette, 0.1 ml of the suspension was transferred to the surface of a blood agar plate (tryptose-phosphate agar base). The inoculum was spread with a glass rod which had been immersed in 95% alcohol and flamed. Spreading was facilitated by revolving the plate on a turntable. After incubation at 37°C for 24 hours the plates inoculated by each method were examined with a wide field binocular microscope ("colony" microscope). Typical colonies were picked and identified by standard confirmatory procedures.

Table I shows the results obtained in respect to 4 bacterial species. In 22 instances one or more of these 4 species was detected by the broth suspension method and not by the dry swab method, while the reverse situation obtained in only 4 instances. These data indicate that the bacterial contents of a swab can be determined better by preparing a suspension in broth than by direct inoculation from the swab.

Further experiments were designed to determine the variations encountered in the broth suspensions. In each experiment, a throat swab was obtained as described above and a broth suspension prepared. Each of 5 plates was then inoculated with 0.1 ml of the suspension. After incubation, the plates were examined with a "colony" microscope and representative colonies picked and identified. The plates were given code numbers so that the examiner was not aware of which plates were inoculated from each suspension. The results of 35 experiments are shown in Table II.

The variations encountered were greater than should arise from a homogeneous suspension, but were less than those observed in similar earlier experiments where mixing was known to have been inadequate. It is apparent that certain organisms were recovered from many of the suspensions with such low frequency that they would have been missed on a single plate regardless of the method of mixing employed.

In order further to evaluate the degree of homogeneity that could be achieved in a bacterial suspension, experiments were carried out with pure cultures. A satisfactory method of

TABLE II.
Relative Frequency of Various Organisms in a Series of Experiments in Which Five Plates Were Prepared from Each Swab.

Swab No.	Readings* Plate No.				
	1	2	3	4	5
Beta-hemolytic streptococci (a)					
1	0	0	0	0	1
9	0	0	0	0	1
15	+	+	+	+	+
20	5	3	1	3	0
22	6	13	3	3	6
30	+	+	+	+	+
33	+	+	+	+	+
H. influenzae (a)					
4	NR	0	15	15	0
5	7	0	0	5	0
8	4	0	13	0	0
12	1	0	0	0	0
13	2	0	0	3	3
14	6	4	0	2	2
15	3	0	5	1	2
16	2	3	0	0	0
18	0	1	0	7	1
21	+	+	+	+	+
22	0	1	1	0	0
23	11	6	12	7	9
24	0	1	0	0	2
28	+	+	+	+	+
29	10	14	15	9	18
30	0	0	3	0	2
31	++++	++++	++++	++++	++++
32	10	5	8	2	14
34	+++	+++	+++	+++	+++
35	+	+	+	+	+
S. aureus (b)					
1	0	11	0	2	2
5	1	0	0	0	0
8	0	0	0	0	+
11	2	1	0	0	0
17	0	8	0	0	0
18	0	0	0	0	1
20	0	0	3	0	0
H. hemolyticus (c)					
11	0	1	0	4	0
12	0	3	0	0	3
13	3	7	12	11	8
14	0	0	1	0	0
16	5	1	3	6	2
19	0	2	0	0	0
20	3	2	4	0	1

* NR = Not Read. Numerals indicate number of colonies of the organisms, which were counted if less than 20.

+
++
+++
++++
} If more than 20 colonies of the organisms; this scale is an estimate of the relative number of colonies of this organism to the total number of colonies on the plate.

(a), (b), (c); plates examined for these organisms were from individuals numbered: (a) 1-35; (b) 1-20; (c) 11-20.

suspending organisms would be one resulting in a random distribution of colony-producing units (whether they be single or clumped organisms) within the resulting suspension. The distribution of such units in a suspension may be determined by making plate counts on measured aliquots from the suspension. The total variation in plate counts should arise from a combination of technical errors or limitations, and sampling variation.

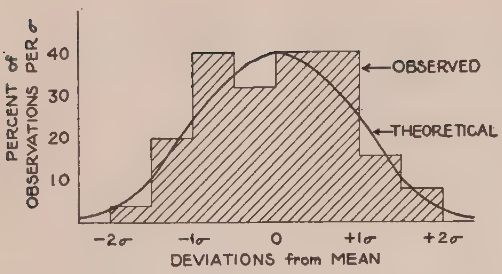

Fig. 1.

Variation in plate counts in a series of experiments in each of which several plates were inoculated with portions of a given suspension.

A loopful of broth culture of *beta*-hemolytic streptococci was transferred to 10 ml of broth and shaken a few times by hand. One loopful was then transferred to 3 ml of broth. The inoculated broth was mixed 20 times with a 5 ml pipette. Five pour plates were prepared using 0.5 ml of the suspension for each plate. Tryptose-phosphate agar with peptic-digested blood was used as the medium. Colonies were counted after the plates had been incubated at 37°C for 24 hours. The experiment was repeated 10 times for a total of 50 plates.

The results of the 10 experiments have been combined into a single experience for the purpose of measuring the variation in successive samples (Figure 1). A theoretical distribution curve is also shown for comparison with the observed results. The theoretical curve shows the results expected if the only source of variation were sampling from a homogeneous suspension.§ The agreement of observed and expected results is close, permitting the conclusion that the suspensions were essentially homogeneous.

Similar experiments were carried out with each of 3 other organisms (*Staphylococcus aureus, Hemophilus influenzae,* and a gram-negative coccus). Variations in plate counts were somewhat greater than expected due to sampling, but not grossly so.

The second technical difficulty investigated—that of the need of visual aid in identifying the colonial characteristics of bacteria—is apparent from Table III. The first 2 columns show the accuracy of naked eye identification of colonies as they appeared on blood agar plates inoculated directly with throat swabs. The last 2 columns show the improvement accomplished by using the wide-field binocular microscope (magnification 15x) to examine plates prepared by the broth-suspension technic.

The advantage of microscopic examination is that it facilitates the accurate differentiation of colonial types and, therefore, leads to the detection of colonies which might have been missed by the naked eye. The fact that it makes possible an increased accuracy of naming colonies is important when a survey is being made for the presence or absence of a particular organism.

The above results indicated that the preparation of a broth suspension from a swab and the use of a microscope in reading plates provided an improved, though not yet ideal, cultural technic, and decreased considerably the errors incurred when the swab was rubbed directly on the culture medium. The data further indicate, however, that the inoculation of only a single plate from the broth suspension, in contrast to multiple plates, considerably decreases the likelihood of determining reliably the presence or absence of an organism of relatively low frequency.

Quantitative growth and nutritional interdependence. The preceding data indicated that a broth-suspension of a single bacterial species can be prepared that for practical purposes is homogeneous, as indicated by growth on the surface of a plate. Similar experiments were then carried out using a mixture of pure cultures of 2 species of bacteria to determine whether or not the growth of each would be quantitative. If such were the case, the colonies of the 2 species should be scattered at random over the surface of the medium. In one set of experiments, *S. aureus* and *beta*-hemolytic streptococci were used; in the second set, *H. influenzae* and *beta*-hemolytic streptococci were employed, because of the nutritional dependence of *H. influenzae* on

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§ If the only source of variation were sampling from a homogeneous suspension the counts in a given experiment should have formed a hypergeometric distribution with a standard deviation equal to the square root of 5/6 of the mean plate count.

TABLE III.
Accuracy of Identification of Colonies with a) No Visual Aid and b) Using the Microscope.

Colony tentatively identified as	(a) No visual aid		(b) Using microscope	
	No. of colonies	% confirmed	No. of colonies	% confirmed
Beta-hemolytic streptococcus	75	40	182	92
S. aureus	20	95	142	96
Pneumococcus	66	5	337	51
H. influenzae	174	53	566	99
H. hemolyticus	94	82	486	97

TABLE IV.
Distribution of Fields of 100 Colonies of a) a Mixture of *Beta*-hemolytic Streptococci and *S. aureus*, and b) a Mixture of *Beta*-hemolytic Streptococci and *H. influenzae*, According to Number of Streptococci per Field.

Deviation* from mean number of Strep. per field	a) Mixture of *beta*-hemolytic streptococci and *S. aureus* No. of fields		b) Mixture of *beta*-hemolytic streptococci and *H. influenzae* No. of fields	
	Observed	Expected	Observed	Expected
–2.0 or more		3.3		1.2
–1.5 to –1.9	1	6.4		2.2
–1.0 to –1.4	12	13.3		4.6
–0.5 to –0.9	28	21.7	8	7.5
0 to –0.4	25	27.8	19	9.6
0 to 0.4	42	27.8	17	9.6
0.5 to 0.9	24	21.7	6	7.5
1.0 to 1.4	12	13.3		4.6
1.5 to 1.9	1	6.4		2.2
2 or more		3.3		1.2
Total	145	145	50	50

* In units of $\sqrt{npq}$

other bacteria, including *Streptococcus hemolyticus*.[19]

In the first group of experiments a 3 ml broth suspension of *S. aureus* and *beta*-hemolytic streptococci was mixed with a 5 ml pipette 20 times. One-tenth ml of this mixture was placed in the center of each of 5 blood agar plates. Each plate was then rotated, using a mechanical turntable, while a sterile glass spreader distributed the inoculum evenly. After incubation at 37°C for 24 hours, the plates were examined with a wide field binocular microscope.

In order to learn whether or not the two organisms were distributed at random over the surfaces of the plates, colonies were counted differentially in fields of 100 colonies each. Forty-five to 50 such fields were counted on the 5 plates. This experiment was repeated 3 times, for a total of 145 fields.

If the colonies of streptococci and staphylococci were distributed at random, the distribution of the number of streptococci per field should be a normal one with a standard deviation of $\sqrt{npq}$ (where p is the proportion of streptococcal colonies, q the proportion of staphylococcal colonies, and n, the number of colonies per field = 100).

Table IV summarizes the differential counts of 145 fields in terms of deviation from the mean[‖] and shows that less variation was observed than expected. For example, the expected number of fields with a deviation

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[19] Davis, D. J., *J. Infect. Dis.*, 1921, **29**, 178.

[‖] For example, in 50 fields counted from one series of 5 plates the average number of streptococci per field was 24.6, and the standard deviation was 4.3 streptococci per field. Eight of these fields showed 27 colonies of streptococci; this was 2.4 colonies more than the average. On a scale of standard deviations these 8 observations fall at $\frac{2.4}{4.3} = +.56$ standard deviation from the mean.

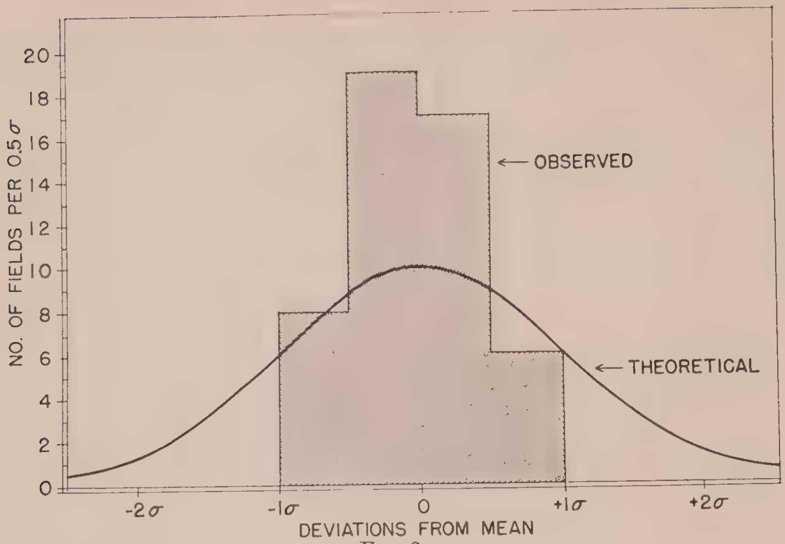

Fig. 2.

% distribution of fields of 100 colonies of a mixture of *Beta*-hemolytic streptococci and *H. influenzae,* according to number of streptococci per field.

of –1.5 standard deviations or greater was 10 while actually only one was observed. Again at the other end of the scale, fields with a relatively high proportion of streptococci were observed less frequently than was expected.

Such lack of variation between the differential counts in various fields is indicative of bias. It suggests that the colonies grew as if the organisms had been placed in relationship to one another in an orderly distribution and not at random, and indicates a relationship, nutritional or otherwise, between the numbers of bacteria of each species which develop into colonies. Probably the presence of one species enhanced the growth of the other.

The second set of experiments was performed in the same manner with a mixture of pure cultures of *H. influenzae* and *beta*-hemolytic streptococci. A total of 50 fields was counted differentially. The lack of variation in differential counts (Table IV and Figure 2) was more marked than with the mixture of *beta*-hemolytic streptococci and *S. aureus,* indicating even a greater dependence of one upon the other.

These observations suggest that the growth on a plate is a resultant of the nutritional inter-relationship of the organisms and the adequacy of the media. The total number of colonies of a given species may be influenced to an undetermined degree by the relative presence or absence of other species. With the large variety of bacteria found in a throat culture this relationship is probably very complex, since not only symbiosis but also antagonism may be involved. Unless these relationships can be circumvented, possibly by selective media or by media adequate for the nutritional demands of all pharyngeal flora, it seems impossible to interpret quantitatively the growth which appears on the usual culture plate.

Thus the absolute or relative growth of colonies on the plate does not necessarily reflect the occurrence and distribution of bacteria in the pharynx. This consideration assumes importance in trying to determine whether organisms normally present in the throat are increased in number in the presence of infection.

Use of a selective medium for detecting beta-*hemolytic streptococci.* Selective media as yet provide a means of detecting the presence of only a limited number of bacteria found in the upper respiratory tract. Because of special interest in *beta*-hemolytic

TABLE V.

Comparison of Two Methods for Detecting *Beta*-hemolytic Streptococci.

	No. positive by Pike method	No. negative by Pike method	Total
Positive by direct method	21	3	24
Negative by direct method	31	195	226
Total	52	198	250

TABLE VI.

Number of Isolations of *Beta*-hemolytic Streptococci from Men Cultured Three Times.

Group and type of streptococcus	1st culture (direct)	2nd culture (Pike)	3rd culture (direct)
Group A, no type*	47	118	48
” A, typable	19	24	7
” B	3	63	1
” C	15	49	27
” F	1	3	5
” G	7	32	12
No group	3	4	2
Not done	1	0	1
Total	96	293	103

* Sera for types 1, 2, 3, 4, 5, 6, 10-12, 11, 14, 17, 18, 19, 22, 23, 24, 26, 28, 29, 30, 31, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 44, 46, and 47 were employed throughout most of the study. Types 8, 9, 13, 15 and 32 were available for part of the period.

streptococci, two studies were made to test the advantage of Pike's selective medium for this organism.[20,21]

In the first study a total of 250 throat swabs was obtained by culturing 125 well soldiers on each of two occasions. Each swab was streaked directly on a blood agar plate, and then treated according to Pike's method. The results are shown in Table V.

The superiority of the selective medium is shown by the fact that *beta*-hemolytic streptococci were found by Pike's technic in 52 of the 250 swabs (20.8%), but in only 24 (9.6%) by the direct plating method. The Pike method detected the organism from 31 swabs which the direct plating method missed, while 3 swabs were positive by the direct method of plating and negative by the Pike method.

In the second study cultures were obtained on 3 occasions from a battalion of approximately 1050 men. The first and third cultures, obtained 3 weeks apart, were rubbed directly onto blood agar plates. The second cultures, taken during the intervening period, were treated by the Pike technic. Grouping of *beta*-hemolytic streptococci was performed by the capillary tube precipitin technic, using commercial rabbit serums and bacterial extracts prepared by Fuller's method.[22] Typing was done by the capillary tube modification of the Lancefield method.[23] ¶

Table VI shows the number of isolations of *beta*-hemolytic streptococci in men who were cultured 3 times. The swabs examined by Pike's method showed an increased number of streptococci in every group. The greatest increase was for Group B, the least for typable strains of Group A. Since the 3 cultures were not taken simultaneously, the results are not strictly comparable. Several facts, however, suggest that the conclusions are valid. The Pike cultures were interposed between the other two during a period when

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[20] Pike, R. M., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 186.

[21] Pike, R. M., *Am. J. Hyg.*, 1945, **41**, 211.

[22] Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.

[23] Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 127.

¶ We are grateful to Drs. Homer F. Swift and Rebecca C. Lancefield for generous supplies of typing sera, capillary tubes, and serum containers; and to Dr. Chester S. Keefer, Director, Commission on Streptococcal Infections, Army Epidemiological Board, for additional sera.

there was no indication of an unusual occurrence of streptococcal infection in the study group. Furthermore, the difference in carrier rate as determined by the two methods in this experiment is compatible with Pike's observations[20,21] and with the results of the cultures of 125 well soldiers (Table V).

Summary. A technic for examining throat swabs which appears to have certain advantages is described. This method involves suspending the material on the swab in broth, inoculating blood agar plates with this suspension, and examining the resultant growth with the aid of a microscope. The experiments which led to the adoption of this technic are presented.

This and other similar technics for determining pharyngeal flora, however, have definite limitations, as indicated by the observation that inoculation of blood agar plates with a mixture of only two bacterial species yielded colonies which were not distributed at random on the surface of the medium, but rather in a manner indicating interdependence of the organisms. Thus, the growth on a plate does not necessarily reflect the relative proportion of each species present in the inoculum.

The value of selective media as a means of detecting organisms present in low frequency has been demonstrated by utilizing Pike's medium for the isolation of *beta*-hemolytic streptococci. The carrier rate for *beta*-hemolytic streptococci was increased from 9.6%, as determined by usual cultural methods, to 20.8% by the use of Pike's method. The increase was greatest for Group B streptococci and least for the typable strains of Group A.

16614 P

Production of Increased Ketonemia in a Normal Dog by Adrenocorticotropic Hormone.*

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Previous work[1] from this laboratory has shown that pure adrenocorticotropic hormone increased the ketonemia of fasted normal rats. The present experiments have been undertaken to establish whether adrenocorticotropic hormone would have a similar effect in a normal dog.

Methods. All the experiments were carried out on one female mongrel dog weighing approximately 15 kg. Two control experiments were done: one in which the dog was given 50 mg of serum albumin, the other in which the dog was given 50 mg of a crude alkaline anterior pituitary extract which had been heated to 100°C for 1 hour. The adrenocorticotropic hormone was prepared according to the previously published method,[2] and 3 experiments were done in which it was given at a dose of either 50 mg or 25 mg.

The dog was fasted 72 hours before each experiment and an interval of at least 2 weeks elapsed between experiments. An initial blood sample was taken and then the hormone or control foreign protein was administered by a single intraperitoneal injection. At 1½, 3, 4½ and 6 hours after the injection additional blood samples were taken. Total blood ketone bodies were determined by the method

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* Aided by grants from the National Institute of Health, RG 409, and The Research Board, University of California.

[1] Bennett, L. L., Kreiss, R. E., Li, C. H., and Evans, H. M., *Am. J. Physiol.*, 1948, **152**, 210.

[2] Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1943, **149**, 413.

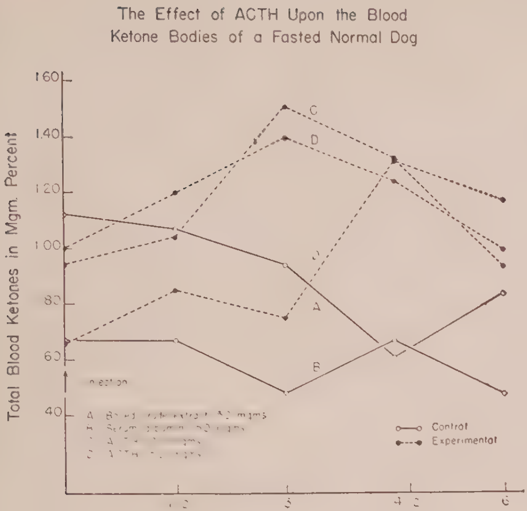

FIG. 1.

of Weichselbaum and Somogyi[3] on 2 ml samples of blood. Blood was taken by venipuncture and in most instances the determination was done in duplicate.

Results. The data from the experiments are presented graphically in Fig. 1. It will be seen that in each case in which adrenocorticotropic hormone was given there was an increase in ketonemia which reached its peak at either 3 or 4½ hours after injection and subsequently declined.

In neither of the control experiments was there a rise in the level of blood ketone bodies. Although the order of magnitude of the change was small it was a large percentage increase in view of the low initial levels. In view of the consistency of the response it was felt to be significant.

Conclusion. Adrenocorticotropic hormone produced an increased ketonemia in a fasted normal dog.

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[3] Weichselbaum, T. E., and Somogyi, M., *J. Biol. Chem.*, 1941, **140**, 5.

16615

A Simple Method for Repeated Bone Marrow Aspirations in Rats.

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During the course of another investigation on the change in the hematopoietic tissues of the adult rat, it became necessary to develop a method for repeated bone marrow aspirations. Sawitsky[1] has perfected a technic for repeated bone marrow aspirations on the guinea pig which was a modification of the Sawitsky and Meyer[2] technic for bone marrow aspiration on cats. Vogel[3] has recently investigated the femoral bone marrow of adult rats, but was unable to obtain marrow without sacrifice of the animal; Cameron and Watson[4] modified Vogel's technic so that repeated aspirations could be made. However, Cameron and Watson's technique requires a surgical incision. This paper will report a modification of the Sawitsky and Meyer technic adapted to rats.

Method. Twenty-six adult rats, (the first generation of a cross breeding of highly inbred Slonaker (Wistar) and Curtis Dunning rats), weighing approximately 200 g were used.

The animals were anesthetized with sufficient nembutal to produce surgical anesthesia of 20-30 minutes duration. The animal was

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* National Cancer Institute Post Doctorate Research Fellow.

† This work was supported in part by the United States Atomic Energy Commission.

[1] Sawitsky, A., and Meyer, L. M., *Blood*, 1948, **3**, 1050.

[2] Sawitsky, A., and Meyer, L. M., *J. L. and Clin. Med.*, 1947, **32**, 70.

[3] Vogel, M., *Am. J. Med. Science*, 1947, **213**, 456.

[4] Cameron, D. G., and Watson, G. M., *Blood*, 1948, **3**, 292-4.

TABLE I.
Differential Cell Count of the Iliac Marrow of Normal Rats.

	Avg	Range
Myeloblast[1]	0.8	0.0- 2.0
Myelocyte	6.7	3.0-13.0
Metamyelocyte	5.1	2.3-10.6
‘‘ring’’ cell	10.9	4.3-19.6
Non seg PMN	4.7	1.6-11.0
PMN	19.2	11.6-30.6
Lymphocyte	20.4	11.0-30.0
Normoblast	24.2	14.6-33.6
Erythroblast[2]	1.3	0.0- 6.6
Megaloblast[3]	0.0	0.0- 0.3
Eos myelocyte[4]	2.0	0.0- 4.3
Eosinophilic ‘‘ring’’	3.6	1.0- 9.0
Eosinophil[5]	1.5	0.0- 4.6
Plasma cell[6]	0.4	0.0- 1.3

[1] Cells of this type were seen in 23/26 animals.
[2] ’’ ’’ ’’ ’’ ’’ ’’ ’’ 23/26 ’’
[3] ’’ ’’ ’’ ’’ ’’ ’’ ’’ 2/26 ’’
[4] ’’ ’’ ’’ ’’ ’’ ’’ ’’ 25/26 ’’
[5] ’’ ’’ ’’ ’’ ’’ ’’ ’’ 25/26 ’’
[6] ’’ ’’ ’’ ’’ ’’ ’’ ’’ 21/26 ’’

grasped in the left hand with the thumb and middle fingers on the proximal part of the right and left femurs, respectively. A 3.0 cm No. 20 gauge spinal needle is held in the right hand so that the right middle finger is free to palpate the right posterior superior iliac spine. The needle is then inserted through the skin to the iliac bone and by a rotary motion is driven through the cortex of the iliac bone. The stylet is removed and suction applied with a dry 20 cc syringe and a drop of marrow aspirated. The syringe and needle are then removed together. The drop of marrow is placed on a clean glass slide and several smears are made directly from this drop. The smears are stained with Wright's stain. Sufficient amount of marrow to do total nucleated cell counts is only occasionally aspirated. It probably is best not to try to do this as the volume removed would be too large and there might be a large admixture with peripheral blood.

Results. From each animal 300 cells were counted. The results are presented in Table I.

Discussion. Vogel has succinctly presented the cellular morphology. No essential difference has been noted between her description of the femoral bone marrow cells and the iliac bone marrow cells observed in this investigation. A few minor points have been observed. Occasionally a large cell in the red cell series —more like the megaloblast than a true erythroblast is seen and has been classified by us as a megaloblast. A somewhat higher percentage of lymphocytes is seen in our aspirations which corresponds more to the values obtained by Cameron and Watson than to Vogel's values. "Kugelhaufen" were seen in most of our smears but were too infrequent to count.

A repeat marrow may be done within 1-2 weeks on the same side. The other iliac bone may be aspirated at any time. Thus by alternating sides, bone marrow samples may be obtained at weekly intervals.

Conclusions. 1. A method for obtaining weekly bone marrow samples of adult rats is presented. 2. The bone marrow obtained from the iliac bone is essentially the same as has been obtained from the femur by previous investigators.

16616 P

Influence of Postnatal Age on Kidney Function of Premature Infants.*

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In the course of an investigation on the development of kidney function, data have been obtained on the influence of postnatal age on kidney function of premature infants and values have been collected for current kidney function tests in premature infants. Measurements were made in 2 groups of premature infants all weighing between 2130 and 2520 g at the time of observation. The ages of infants in the first group (Group A) ranged from 3 to 13 days; those in the second (Group B) from 49 to 107 days.

Methods. The clearance procedures and chemical methods have been described previously.[1]

Results. Mean values for inulin, urea, mannitol and para-aminohippurate (PHA) clearances and PAH tubular maxima in 17 well female premature infants are shown in Table I.† Proctocols for each subject are included in the table. The individual values in Table I are calculated without correction as UV/P. For comparison with adult values, the means for each group are corrected for surface area. With this correction, the inulin clearances are 41% (Group A) and 58% (Group B) of normal adult values; the PAH clearances 25 and 34% and PAH tubular maxima 17 and 27%, respectively.

It may be seen from the data in Table I that although some overlapping occurs, mean values for all measurements are higher in the older than in the younger infants. A marked increase in clearances is noted only in the oldest infant, ST, whose corrected inulin clearances are actually within the normal adult range. For the most part, however, the magnitude of the differences between the two groups is surprisingly small. Examination of values for individual infants gives emphasis to this latter fact: for example, at 12 days of age, PI "A" had higher values for all determinations than WI had at 66 days and higher PAH clearances than KO had at 95 days of age.‡

The C_{IN}/C_{PAH} ratios are higher in these infants than in adults. A high ratio in early infancy falling to adult values during the first few months of life has been reported.[2,3] It is of interest that the C_{IN}/C_{PAH} ratios in the two groups of infants reported here are almost identical.

Discussion. Cessation of new glomerular formation in the kidneys of fetuses and infants weighing between 2100 and 2500 g has been reported to be dependent on the size the fetus has attained rather than related to the fact that birth has occurred.[4] Our data on premature infants of the same

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service. Mannitol and para-aminohippurate were supplied by Sharp & Dohme, Inc.

[1] Barnett, H. L., Hare, K., McNamara, H., Hare, R. S., *J. Clin. Invest.*, in press.

† The detailed data for inulin and urea clearances in Group A have been presented in Table Ib of a previous report.[1]

‡ Part of this wide scatter may be attributed to inaccuracy of body weight as an index of gestational age. However, the difference between the two groups in range of birth weights and postnatal ages is so great that variations in maturity related to multiple births, race, or other factors, would be minimized.

[2] Rubin, M. I., Bruck, E., and Rappaport, M., Proc. Fifth International Congress of Pediatrics, 1947, p. 99.

[3] West, J. R., Smith, H. W., and Chasis, H., *J. Pediat.*, 1948, **32**, 10.

[4] Potter, E. L., and Thierstein, S. T., *J. Pediat.*, 1943, **22**, 695.

TABLE I.
Protocols and Data on Renal Clearances in Premature Infants.

Subject	Day of observation			Birth wt, g	Clearances* ml/min				PAH tubular maxima mg/min	$\frac{C_{IN}}{C_{PAH}}$
	Age, days	Wt, g	Ht, cm		Inulin	Mannitol	Urea	PAH		
					Group A					
BA‡	3	2130	46	2180	3.48	3.07	1.77	11.86	0.61	.29
GU†	4	2276	46	2300	5.47	4.64	—	16.46	1.85	.32
RA‡	9	2380	47.5	2468	4.60	3.99	3.28	13.75	0.77	.33
PI‡	9	2216	47	2118	5.49	4.46	3.34	13.92	1.17	.42
PI‡	12	2320	47	2066	6.59	6.09	4.06	20.10	2.31	.32
MA	12	2190	46	2182	4.06	3.98	2.98	15.82	1.44	.27
RO	13	2164	45	2090	4.36	3.97	2.90	9.91	—	.42
PA	13	2164	—	2100	3.29	3.12	2.25	—	0.69	—
Mean: uncorrected					4.67	4.17	2.94	14.55	1.26	.34
Mean: per 1.73 M^2					47.8	42.7	30.1	148.5	12.9	—
					Group B					
MC	49	2300	46	1360	6.38	5.64	3.75	22.09	2.28	.31
LE	51	2360	47	1340	5.78	5.13	4.45	16.05	1.39	.31
PR‡	53	2470	45.3	1310	7.02	6.81	4.73	22.88	1.47	.30
MB	54	2156	43.5	1018	7.33	6.19	4.52	18.77	1.62	.41
KO	55	2404	46	1175	6.79	5.41	4.85	18.08	1.67	.33
WI	66	2440	47	1240	5.09	—	2.67	12.77	1.56	.42
MN	73	2420	44	960	6.06	—	2.96	20.89	1.20	.29
KU	95	2510	47	740	6.77	—	4.74	19.20	2.84	.37
ST†	107	2520	45	820	11.09	—	8.04	35.02	5.31	.34
Mean: uncorrected					6.92	5.84	4.52	20.64	2.15	.34
Mean: per 1.73 M^2					67.4	58.0	44.0	200.7	20.8	—

* Each value represents the mean of 3 to 10 clearance periods.
† Negro.
‡ Twin.

weight range show some increase in kidney function related to postnatal age. However, judging from the rate of development of kidney function in larger infants[3,5] and in rabbits,[6] the magnitude of the increase observed in our older infants may be less than would be expected in larger infants. In addition, the C_{IN}/C_{PAH} ratios did not change in our infants with increasing clearances. These latter findings suggest that development of kidney function in infants weighing up to 2500 g may be influenced by a gestational factor not operating in larger infants. A more complete description of these relationships requires direct comparison of these results with similar data on full term infants. Such observations are planned.

Conclusions. Mean values for inulin and PAH clearances and PAH tubular maxima corrected for surface area in premature infants ranged from 17 to 58% of normal adult values. Greater postnatal age appears to increase the rate of development of kidney function in premature infants weighing between 2100 and 2500 g although to a less extent than might be expected in full term infants.

We are indebted to Flora Hurwitz, R.N., for nursing and technical assistance.

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[5] Gordon, H. H., Harrison, H. E., and McNamara, H., *J. Clin. Invest.*, 1942, **21**, 499.

[6] Williamson, R. C., and Hiatt, E. P., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 554.

16617

Strains of the Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico.

Ian A. Galloway, W. M. Henderson, and J. B. Brooksby.
(Introduced by R. E. Shope.)

From the Research Institute (Foot-and-Mouth Disease Research Committee), Pirbright, Surrey, England.

The present series of papers deals with studies on strains of the virus of foot-and-mouth disease from outbreaks in Mexico. The work was undertaken at the request of the Bureau of Animal Industry of the United States Department of Agriculture on behalf of a joint Mexican-American Commission to whom all the results recorded here have been reported.*

During the experiments recourse has been made to many new methods that have been developed at this institute, to which some preliminary reference should perhaps be made. The necessity of having a quantitative appreciation of a biological problem is becoming increasingly apparent especially in approaching problems of immunity. No great reliance can be placed on the results of "field" vaccination experiments, for example, in estimating the real value of an immunizing agent as it is not possible to analyse satisfactorily the many factors involved. This can be done only under controlled experimental conditions. Again before satisfactory interpretations can be made of the results of experiments carried out under controlled conditions it is necessary to have reliable "standard methods."

The main program of the scientific staff of this Institute for the last eight years has been directed with this object in view. As a result it is now possible to measure the potency of virus suspensions of bovine origin such as are used for vaccine production by a method of titration involving the simultaneous inoculation of a number of serial dilutions of virus in the bovine tongue.[1] Sufficient observations are made for the calculation of the 50% end-point. This method guarantees the greatest accuracy commensurate with the use of a very limited number of animals.

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* The authors wish to express their thanks to the joint Mexican-American Commission (Lic. Oscar Flores (Mexico) and Dr. M. S. Shahan (U.S.A.), co-directors; Dr. B. T. Sims, Chief of the Bureau of Animal Industry, and Dr. Fernando Camargo, Director of the Laboratories of the Mexican Ministry of Agriculture and Livestock Industry, for the opportunity to examine the material. Their thanks are also due to Dr. H. W. Schoening in charge of the Pathological Division (B.A.I.), for his continued interest during the progress of the investigations.

In serum neutralization tests correlated with electrophoretic studies and the development of methods for concentrating antibodies it has been shown that by application of the above titration method it is possible to estimate with a greater degree of accuracy than was formerly attainable, the neutralizing and protective properties of bovine antisera and to detect low levels of antibody.[2] For quantitative estimates of this sort it is obvious that there must be basic information on the relative susceptibility of the test animals employed. In the case of foot-and-mouth disease where cattle are the experimental animals of choice for the study of virus of bovine origin, this susceptibility must vary within wide limits according to the source. There is on the one hand the stock of a country in which the disease is not endemic and in which variations in susceptibility may be attributable to other factors such as age, condition and breed and on the other hand the stock of a country in which the disease is endemic. In this case the "clean history" of the experimental animals is an extremely doubtful factor. This difficulty has been overcome in some countries *e.g.* Holland, by the importation of test animals from a country which is free from the disease.

It should be emphasized that in all the investigations at Pirbright including those under discussion, cattle with a "clean history" and a relatively uniform level of susceptibility have been employed. While this is indispensable in estimates of virus potency and antibody levels it is equally important in the true appreciation of the limitations of tests for the non-infectivity of vaccines and in estimating the immunizing value or potency of vaccines.

Reference has been made by one of us (I.A.G.) at divers meetings to the results obtained at this Institute in developing standard methods. These included meetings held under the auspices of the Office International des Epizooties in Paris in May, 1947 and in Berne in September 1947,[3] the 4th International Congress of Microbiology in Copenhagen in July, 1947 (see Proceedings) and a meeting organized by F.A.O. held at Pirbright in September, 1947. In addition to the methods mentioned above extensive observations carried out on several thousand cattle under controlled experimental conditions have shown that it is possible to estimate the relative potency of different vaccines in contact exposure tests by administering graded doses of vaccine. Results having statistical significance can be obtained, based on the calculation of 50% end-points provided that groups of an adequate number of vaccinated and untreated control cattle are employed. In such circumstances it has been demonstrated that different strains of virus have different immunogenic properties against a strictly homologous virus infection (*i.e.* infection with the same strain) with the implied necessity of selection of suitable strains for vaccination. At the meeting in Copenhagen and again at the 4th International Congresses of Tropical Medicine and Malaria held in Washington in May 1948 preliminary reports were made by one of us (I.A.G.) of some of the experiments, the results of which are embodied in this series of papers. One of the points connected with the immunity problem which has received particular attention has been the difference in antigenic behavior of strains of virus within a main immunological type group.

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[1] Henderson, W. M., Thesis, University of Edinburgh, (in press as an Agricultural Research Council Report), 1945.

[2] Brooksby, J. B., Thesis, University of London, (in press as an Agricultural Research Council Report), 1946.

[3] Galloway, Ian A., *Bull. Off. Internat. Epizoo.*, 1947, **27**, 520.

Since these matters were reported another line of approach has been introduced for the detailed study of strains of virus, the application of the complement fixation test. Reference has already been made by one of us[4] to the development at this Institute of the technic on a quantitative basis.

It is encouraging that already this necessity of developing standard methods on which so much insistence has been placed by the authors is now being widely recognized and their precepts are coming to be adopted more generally.

Paper II[5] deals with the identification of 13 samples of virus material from Mexico and the accumulated evidence justifies the conclusion that only the virus of foot-and-mouth disease was present in any of the virus samples examined and all the methods employed for the determination of the immunological type involved indicated that all the strains could be placed in the Vallèe A group.

There should be some basic information on the pathogenicity and invasiveness of the virus strains concerned before immunity studies are pursued and unless this is forthcoming it is impossible to interpret the results of vaccination experiments correctly. Paper III[6] summarizes the observations on these characteristics of the Mexican strains of virus. Evidence is produced to illustrate the low invasiveness of these strains. The question has been discussed at some length there as it is of great interest not only because of its epizootiological significance but also in the interpretation of the results of vaccination experiments. Further points merit elaboration here. It is often exceedingly difficult if not impossible to secure any information of real value on the origin of a particular outbreak or epizootic of foot-and-mouth disease and much of the evidence advanced does not proceed beyond the realms of speculation. In this respect the present epizootic in Mexico is no exception.

It is not known how strains of low invasiveness arise. We do not even know whether low invasiveness as determined by tests on a particular breed of cattle of fairly uniform susceptibility under a given set of conditions would be equally demonstrable if observations were made on another breed of different age and conditions in a different climate.

It is known that some species adaptation of the virus appears to occur, for strains of virus of porcine origin sometimes show poor infectivity for cattle. One of us (J.B.B.) has studied such a strain recently and while it spread readily amongst swine it did not do so from swine to cattle. The supposition that the Mexican strains of virus may have originated from swine would appear to be discounted since as has been reported in Paper II,[5] strain MP (23.12.46) showed no special affinity for swine. On the other hand in countries in Africa, South America and Asia where the disease is endemic and where also susceptible wild animals of different species may roam as in Mexico (*e.g.* deer, wild pigs, goats, llamas, tapirs, porcupines etc. as well as small susceptible rodents of different kinds) it does not seem to be a remote possibility that the disease may spread among these and then return to infect cattle in a modified form due to passage through and perhaps some adaptation to species other than cattle. Where such conditions prevail *i.e.* a virus of low invasiveness is spreading and leaving in its wake animals with a varying degree of immunity; are they not conducive to the development of "variants" and also to the persistence and smouldering of the disease perhaps in a mild atypical form which at certain periods might not be readily recognizable as foot-and-mouth disease? If a virus spreading in this way reaches say "closed" dairy herds of a more susceptible breed of cattle *e.g.* cattle of European origin, this might provide the circumstances and necessary medium for an enhancement of the invasiveness of the strain and a "flare up" which first attracts attention to the existence of the disease.

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[4] Brooksby, J. B., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 254.

[5] Brooksby, J. B., Henderson, W. M., and Galloway, Ian A., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 64.

[6] Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 66.

Turning to the problem of the interpretation of experiments on vaccination it is apparent from what has been outlined elsewhere[6,7] that strains of low invasiveness create added difficulties. When in contact exposure tests a considerable number of untreated susceptible control cattle fails to develop visible lesions but on the other hand becomes immunized as a result of infection it is exceedingly difficult to know to what extent the resistance exhibited by the nonreactors in the vaccinated groups can be attributed to the vaccine and to what extent to the low invasiveness of the strain of virus, unless the number of animals in each group is increased beyond the limits of practicability. How much more important this problem becomes in countries where it is difficult to procure cattle of uniform susceptibility! It is known that in countries in which the disease is endemic and where it is not the practice to select animals according to age, breed and condition, it is not uncommon to find quite a number of cattle which will not react even to intradermal inoculation of the tongue with massive doses of strains of virus of high invasiveness. It would therefore not be surprising if the number of non-reactors to such inoculations was not materially increased when strains of low invasiveness such as the Mexican strains are under investigation. The significance of the virus-host relationship is further illustrated by the facts which emerge from the observations on the titration of two of the Mexican strains of virus, MP and M.1 (in suspensions prepared from the vesicular tongue lesions or in the circulating blood) referred to in Paper III.[6]

It is obvious that unless there is awareness of the influence of the factors discussed above there can be no satisfactory approach to a discussion of what are sometimes glibly referred to as "international standards".

When in vaccination experiments there are indications that the invasiveness of the strain of virus under investigation is low and the susceptibility of the cattle is nevertheless relatively and uniformly high, it is possible as has been recorded in Paper VI[7] to use another method of estimating the potency of a vaccine. This is dependent on observations on the occurrence or non-occurrence of secondary lesions on the lips or feet or elsewhere following upon the inoculation of virus intradermally into the tongues of groups of vaccinated and control cattle. As in contact exposure tests little reliance can be placed on observations on too small groups of cattle for as will be seen from the results secondary lesions sometimes do not develop in the untreated control cattle.

Further work is being done to find out the correlation between the results in potency tests of vaccination with graded doses of vaccine followed by exposure to infection by contact and similar potency tests in which the vaccination with graded doses of vaccine is followed by exposure to infection by inoculation of virus intradermally into the tongue or by other routes.

Particular attention must be drawn to the results of experiments which are recorded in Papers IV,[8] V[9] and VI[7] illustrating the antigenic behavior of the Mexican strains of virus MP and M.1 in relation to 119 the Pirbright stock strain of virus of the main Vallée A immunological type.

It is perhaps as well to deal first with the observations which were made in vaccination experiments with virus strains MP (Mexican) and 119 (Pirbright). When it was found that the first samples of virus sent from Mexico including strain MP were of the Vallée A immunological type, an experiment was set up to determine whether a vaccine prepared from strain 119 (Vallée A) would give as good protection to cattle exposed to infection with the Mexican strain MP as to cattle exposed to infection with strain 119. From observations as yet unpublished carried out under strictly controlled experimental conditions on 320 vaccinated cattle and 116 untreated control cattle in a series of experiments, it has been proved that strain 119 is of high immunogenicity against strictly homologous virus infection (50 per cent end-point protection dose = 8 cc). As will be seen from the results of the recorded experiments in Paper VI,[7] a vaccine prepared from strain 119 did not, when administered in equivalent dose, give the same measure of protection against infection with strain MP as it did against infection with strain 119.

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[7] Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 77.

[8] Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 70.

[9] Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 74.

When this result was obtained an examination of the two strains was made in cross serum neutralization tests in cattle, Paper V,[9] and it was found that bovine anti-sera had a greater neutralizing effect on strain homologous than on strain heterologous virus. In complement fixation tests also, Paper IV[8] differences were apparent in the antigenic behaviour of strain MP and strain 119 when anti-sera from guinea pigs infected with the respective strains were employed.

Cross serum neutralization and complement fixation tests were then carried out with strains MP and M.1 and differences were exhibited also in the antigenic behaviour of these strains both in relation to strain 119 and to one another. Since strains MP and M.1 appeared to differ from one another in their behaviour in complement fixation and serum neutralization tests an investigation of their antigenic behaviour in cross immunity or vaccination experiments was made. As will be seen in Paper VI[7] the results of these preliminary tests were not conclusive although their trend suggested some difference between strain MP and M.1. It is conceivable that in similar experiments in which groups of cattle are tested by the same method after injection with smaller graded doses of vaccine it may be possible to bring out greater differences in the antigenic behaviour of these strains. It is obvious that further work is necessary to clarify the position and that all due caution must be exercised in drawing conclusions. However, even at this stage, there is justification for the statement that the results of the examination of the antigenic behaviour of the Mexican strains of virus has strengthened the evidence for the occurrence of "variants" within the main Vallée A immunological type group. The use of the term "variant" is one of convenience only and in this instance is for the purpose of describing some observed difference in the antigenic behaviour of strains of virus which are so similar in their behaviour in general immunological typing tests that they can clearly be classified only in one particular type group. This class of "variant" is distinct from others apparently more complex which has been described by Traub and his co-workers[10,11] as the result of complement fixation tests. These latter variants show some partial relation to other immunological type groups. There appears to be correlation in some instances at least between the differences brought out by the three methods of study of antigenic behaviour but a considerable amount of work is now necessary to examine as many strains of virus as possible to see whether this is universally the case. Particular attention is being paid to the strains of virus regularly employed for vaccine production in the different European centers. It would be tempting to assume that because agreement in the results of complement fixation, serum neutralization and cross-immunity tests has been observed in the experiments recorded here it would be possible to forecast the results of vaccination experiments with a strain of virus from the results of the other two simpler tests. It is premature to assume that this would be so. It would be reasonable to suppose that some of these antigenic differences may be quantitative and that the limited range of antigenicity of certain strains may be compensated for by increasing the amount of antigen administered in vaccination or again some of the difficulties may be overcome by the selection of strains of good immunizing power and of a wide range of antigenicity. The real significance of the occurrence of these "variants" within an immunological type group has yet to be assessed. What is strongly suggested

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[10] Traub, E., and Rodriguez, R., *Zbl. Bakt. Abt.* 1, Orig., 1944, **151**, 380.

[11] Traub, E., and Möhlmann, H., *Berl. und Münch. tierärztl. Wschr.*, 1946, **1**, 1.

is that these observations cannot be ignored in immunization procedures since Traub and Möhlmann[11] while working in Europe recovered some similar Vallée A "variants" from some cattle which developed the disease although they had been vaccinated in the field with a stock bivalent vaccine in which two strains of virus, one of the Vallée O type, and the other of the Vallée A type were incorporated. The cattle in these "vaccine breaks" had been vaccinated with a Schmidt-Waldmann aluminum hydroxide vaccine.

Their method of approach to the problem was different from that of the present authors in that they came across these "variants" while carrying out complement fixation tests. They did however add a rider to the effect that although the late war had prevented them from being in a position to place their observations on record, they had found that the Vallée A strain of virus used for vaccine production was not so immunogenic against the other "variants" as it was against itself and the same held true for the behaviour of the other "variants."

It is apparent, and this was stressed at the 4th International Congress of Microbiology in Copenhagen, that the position with regard to strains of virus of foot-and-mouth disease is similar to that which appears to exist with strains of influenza virus *i.e.* "variants" occur within a main immunological type group. Where workers on foot-and-mouth disease have an advantage over those engaged on the influenza problem is that although for obvious reasons progress is bound to be slower, the strains of virus can be studied in the host from which they were recovered and cross immunity vaccination experiments involving exposure to infection with a selected strain of virus can be carried out on cattle in which the disease occurs under natural conditions.

In the present series of experiments the Mexican strains MP and M.1 and the stock strain 119 were all strains of bovine origin. The serum neutralization tests were made with bovine antisera and these and the cross immunity tests were carried out in cattle. Until some means is devised of overcoming the difficulties encountered in using bovine antisera in complement fixation tests, virus strains have to be adapted to guinea-pigs for the preparation of antisera for this purpose. It would be preferable to avoid this procedure. As a result of the observations recorded above it was recommended to the Bureau of Animal Industry of the U. S. Department of Agriculture for the benefit of the joint Mexican-American Commission that until further information became available on the range of immunogenicity and specificity of other Vallée A type strains of virus it would be preferable to use Mexican strains of virus for vaccinating in Mexico (Results of experiments in which cattle injected with vaccines prepared from Mexican virus strains were subsequently exposed to infection with the same strains are recorded in Paper VI[7]). When again from the results of experiments some antigenic differences appeared to exist between the Mexican strains MP and M.1 it was recommended that until further information was obtained both these strains should be incorporated in vaccines for use in Mexico, in fact a bivalent vaccine incorporating MP and M.1 should be employed. The position is however in a constant state of flux and it must be reviewed from time to time.

This is neither the time nor the place to discuss fully the observations recorded in this series of six papers in relation to the control of foot-and-mouth disease. The procedure must be governed by the conditions obtaining. Attention might however be drawn to the more obvious implications. Every effort should be made to prevent foot-and-mouth disease becoming endemic in a country. This can be accomplished in certain exceptional circumstances by a "stamping-out" policy alone involving the early and rapid slaughter and disposal of carcasses by burial or burning and the application of a "cordon sanitaire" including police and quarantine measures.

If vaccination has to be introduced, owing to the failure or inapplicability of a "stamping out" policy, either alone or in combination with slaughter in zonal control measures or on a more extended basis then rapidity is again the key to the situation and every effort should be made to induce as high a grade of immunity in as many susceptible animals as possible in as short a time as possible.

Whatever the class of vaccine to be employed, there must be information obtained on the strain or strains of virus responsible for the outbreaks.

Furthermore a phase has now been reached in research on the disease which points to the necessity of "standard methods" such as those referred to above so that as highly effective vaccines as possible can be produced. This involves the selection of strains of good immunogenicity and of as wide a range of group antigenicity as possible. The last word on vaccination against foot-and-mouth disease has not yet been said. If when the disease is already, or should owing to untoward circumstances, become endemic new aspects of the control problem arise but this theme cannot be developed here. However something should be said about one or two points of interest. Under epizootic or endemic conditions is it not likely that some reported cases of "reinfection" in herds which were known to have passed through the disease only a relatively short time before may be due to infection, with the same strain of virus, of cattle which escaped the disease before, or at least acquired only a low grade of immunity due to the low invasiveness of the strain? Another possibility is that a strain "variant" may have arisen. It is often assumed without further investigation that such cases of so-called "reinfection" are due probably to another immunological type. In at least one specific case of reported "reinfection" during the Mexican epizootic such a belief was not substantiated by the examination of the virus sample collected. This aspect requires further study.

Examination of samples of virus from outbreaks during the course of an epizootic has of necessity in the past been very fragmentary. The observations recorded here suggest strongly the advisability of a more detailed and continued examination of as many virus samples as possible. This would contribute to a better understanding of the epizootiological picture and the sequence of events. Indeed it would be essential if effective control and possibly ultimate elimination of the disease was the aim in view.

Nothing has been said about the possibility of the occurrence of other vesicular diseases such as vesicular stomatitis or vesicular exanthema at the same time in areas where foot-and-mouth disease is spreading but that is another question.

The problem of the attempted control of foot-and-mouth disease by procedures involving vaccination is obviously more complex in some cases than is generally appreciated but not so complex that continued scientific endeavor may not introduce considerable improvements in many directions. It should be apparent also that in applying methods of control involving vaccination, continual guidance and indeed direction of the program must come from the laboratory.

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Strains of Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Identification.

J. B. Brooksby, W. M. Henderson, and Ian A. Galloway.
(Introduced by R. E. Shope.)
From the Research Institute (Foot-and-Mouth Disease Research Committee), Pirbright, Surrey, England.

These observations were made during the examination of samples of virus which were recovered from cattle during an epizootic in Mexico. It was reported that foot-and-mouth disease existed in Mexico in the last quarter of 1946 and now in June, 1948, fresh outbreaks of the disease are still occurring. Up to the present 13 separate samples of virus from Mexico have been examined at this Institute and as will be seen from the results recorded all of them could be classified in the main Vallée A immunological type group. After identification these virus samples were termed strains of the virus of foot-and-mouth disease. For the purpose of future reference a list is appended with the dates of collection and reported geographical origin:

MP—Puebla, Dec. 23, 1946
MPB—Puebla, Jan. 1, 1947
MC—Camarones, Jan. 21
MSL—San Lorenzo, Jan. 31
M.1—Jalisco, Oct. 7
M.2—Cuerrero, Oct. 11
M.3—Toluca, Sept. 20
M.4—Lerma, Sept. 30
M.5—El Salto, Jalisco, Nov.
M.6—El Bajo, Jalisco, Mar. 19, 1948
M.7—Guzman, Apr. 12
M.8—Zapotiltic, Apr. 13
M.9—Zapotiltic, Apr. 13

So far the antigenic behavior of only 2 of the virus strains MP (23.12.46) and M.1 (7.10.47) have been investigated in some detail but further work is proceeding on the Mexican strains of virus and the results will be reported later.

These studies on antigenicity which are reported in other papers of the present series include complement fixation, serum neutralization and vaccination experiments.

Identification of infective agent and general immunological type classification.

1. *Animal Species Susceptibility.*

(a) Inoculation of cattle.

The respective inoculation of suspensions of the thirteen samples of material received intradermally into the tongues of cattle has produced in every case a generalized vesicular disease resembling, in its general characteristics, foot-and-mouth disease.

(b) Inoculation of horses and donkeys. Suspensions of some of the samples of material received were inoculated intradermally into the tongues of horses and donkeys as follows:

M.1—2 horses and 2 donkeys
M.2—2 horses
M.3—2 horses
M.4—2 donkeys
MP—2 donkeys

and contrary to what might have happened if the virus of vesicular stomatitis had been involved, no lesions were observed in any instance.

(c) Inoculation of swine. Two swine were inoculated intramuscularly with a suspension of the MP strain of virus after passage in cattle, one showed a vesicular lesion on the snout on the third day and on the feet on the fourth day and the other failed to develop lesions. A third pig inoculated by scarification of the snout and intradermally into the tongue with a suspension of the MP strain of virus showed no lesions.

(d) Inoculation of guinea-pigs. It was possible to infect guinea-pigs with suspensions of virus samples MP, MPB, MC, MSL, M.1 and the 9 strains to M.9 by inoculation intradermally into the pads of the hind feet and a generalized vesicular disease developed after adaptation to this species. Reference is made later to typing tests in guinea-pigs.

2. *Complement Fixation Tests.* These

have been made with stock type foot-and-mouth disease and vesicular stomatitis guinea-pig antisera. Tests involving the use of "Indiana" and "New Jersey" vesicular stomatitis stock type antisera were made with the 10 "virus samples" MP, and M.1 to M.9 and all yielded negative results. These vesicular stomatitis type antisera had been tested against their respective agents and had been proved type specific.[1]

Complement fixation tests involving the use of Vallée A, Vallée O and Waldmann C stock type foot-and-mouth disease antisera were carried out with all the 13 virus samples and positive results were obtained in every case, except one (MPB), with the Vallée A antiserum and not with the Vallée O and Waldmann C antisera.

The complement fixation tests were carried out in most cases with first passage material, collected from the tongue lesions of inoculated cattle, as antigen but in one instance at least (MP) a positive result was recorded when a suspension of the original material sent, in 50% glycerine phosphate at pH 7.6, was employed as antigen. Negative results were recorded in the tests with MPB both on the original sample and on the first passage cattle material but the matter was not pursued further as typing tests in guinea-pigs and in cattle had already indicated that MPB could be placed in the Vallée A type group of the virus of foot-and-mouth disease.

3. *Differential Filtration.* Strains MP and M.1 after adaptation to guinea-pigs were examined respectively by fractional filtration through graded "gradocol" (Elford) collodion membranes and the end-point of 25 mμ enables a size value of 8 to 12 mμ to be attributed to both. This is the size value previously recorded for all strains of foot-and-mouth disease virus examined. The size of the virus of vesicular stomatitis is about 8 times as great, *viz.* 70-100 mμ.

4. *Inoculation into developing hen eggs.* It was shown that MP virus would not survive when inoculated on the chorio-allantoic membranes of developing hen eggs incubated at temperatures of 35°C to 39°C (*cf* the virus of vesicular stomatitis which can be propagated in eggs under these conditions). Strain M.1 behaved similarly.

5. *General Immunological typing in guinea-pigs.* Five of the Mexican virus strains MP, MPB, MC, MSL and M.1 after adaptation to guinea-pigs were examined in cross immunity tests with standard type guinea-pig strains 1 (Vallée O) GB (Vallée A) and GC (Waldmann C) and as a result all 5 were classified as of the Vallée A type of the virus of foot-and-mouth disease.

6. *General immunological typing in cattle.* In all 28 cattle recovered from infection with known Vallée A type strains of the virus of foot-and-mouth disease have been inoculated with 8 of the virus strains under discussion *viz.* MP (8), MPB (4), MC (4), MSL (4), M.1 (2), M.2 (2), M.3 (2) and M.4 (2). With the first 5 strains the inoculations were made intramuscularly and none of the 22 cattle inoculated showed any lesions; 14 of these cattle were recovered from infection with the Pirbright Vallée A type stock cattle strain 119 and 8 had recovered from infection with an Argentine (A. Cor) Vallée A type cattle strain of the virus of foot-and-mouth disease. On the other hand 9 control susceptible cattle similarly inoculated respectively with the different virus samples MP (2), MPB (2), MC (1), MSL (2) and M.1 (2) all developed a generalized infection. With the remaining 3 virus strains M.2, M.3 and M.4 cattle recovered from infection with Vallée A type strain 119 were inoculated, 2 cattle for each strain, intradermally into the tongue with suspensions and although local reactions at the site of inoculation were recorded in 5 out of the 6 animals, no secondary lesions followed on the feet and elsewhere whereas a generalized disease involving these sites was observed on all of 6 control susceptible cattle similarly inoculated. In addition it may be mentioned that the relationship as regards the main group immunological typing (Vallée A) of the Mexican virus strains was further illustrated by the fact that of 8 cattle recovered from infection with the Mexican strain MP and subsequently inoculated either intramuscularly (M.1) or

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[1] Brooksby, J. B., PROC. SOC. EXP. BIOL. AND MED., 1948, 67, 254.

intradermally into the tongue (M.2, M.3 and M.4) with 4 other Mexican strains of the virus, none developed any lesions.

Furthermore a limited number of tests were made by inoculating Mexican strains of virus into cattle recovered from infection with Vallée O and Waldmann C type strains of the virus of foot-and-mouth disease and in all of these a generalized foot-and-mouth disease infection resulted.

On perusal of the accumulated evidence it will be seen that none of it would suggest that any infective agent other than the virus of foot-and-mouth disease was present in any of the 13 samples of material collected from cattle in Mexico and forwarded to Pirbright for examination. Moreover each of the 13 strains could be clearly classified in the main Vallée A immunological type group of the virus under discussion. The examination of these Mexican strains of virus involved inoculation of cattle, horses, donkeys, swine, guinea-pigs and developing hen eggs, fractional filtration experiments, complement fixation tests and cross immunity typing tests in guinea-pigs and cattle against stock type strains of the virus of foot-and-mouth disease and it was therefore much more exhaustive than that usually made of "unknown" samples of virus.

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Strains of Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Pathogenicity and Invasiveness.

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(Introduced by R. E. Shope.)

From the Research Institute (Foot-and-Mouth Disease Research Committee), Pirbright, Surrey, England.

A report on the identification of samples of virus received from Mexico has been made.[1]

Before referring to the general effects of the Mexican strains of virus in cattle exposed to infection by inoculation, by contact with diseased cattle, or by the nasal instillation of virus, something should be said about the animals employed in these experiments.

Foot-and-mouth disease is not endemic in Great Britain but is introduced at irregular intervals, outbreaks occurring sporadically. Since the policy of control is by "stamping-out," that is, elimination of infected and "in-contact" animals by slaughter and burning (or under the exigencies of war, by burial) along with the rigorous application of other disinfection and "stand-still" orders, it is possible only to purchase cattle with a "clean" history as regards foot-and-mouth disease. The cattle used in these experiments were Devon steers between 1½ and 2 years old, in good condition and weighing between 600-800 lbs, and they could be considered as relatively "standard."

1. *Intradermal inoculation into the tongue.* In all 296 cattle have been inoculated with centrifuged suspensions or stock filtrates of the 13 strains of Mexican virus. 184 were inoculated with MP virus, 80 with M.1 virus, and the remaining 32 with the other 11 strains. 293 of these 296 reacted well, with local lesions at the sites of inoculation and generalized or secondary lesions at some or all of the usual sites of predilection, feet, and lips, in all cases except 2. These 2 were control animals inoculated with filtrates in an experiment described elsewhere.[2]

There were 3 cattle which failed to show lesions at the sites of inoculation. These had been inoculated with similar virus material to that which had produced the disease in other cattle. One of these however subsequently developed lesions at sites other than those inoculated, probably resulting from infection by contact with its box companion which had reacted. The other 2 developed local lesions on the tongue when re-inoculated with the Pirbright stock cattle strain 119 of the Vallée A type.

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[1] Brooksby, J. B., Henderson, W. M., and Galloway, Ian A., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 64.

[2] Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 77.

Such occasional failures to react to intradermal tongue inoculations of relatively large doses of virus have been observed with other strains of the virus of foot-and-mouth disease.

In general therefore Mexican strains of virus produce infection in the Pirbright cattle with the same degree of regularity as other strains when inoculated intradermally into the tongue.

However, there appears to be a tendency for delayed appearance of the secondary lesions. Moreover, although the presence of these secondary lesions on all 4 feet and sometimes the lips has been observed in a considerable number of cattle inoculated intradermally into the tongue with Mexican strains of virus, in many animals generalization has been incomplete and limited as regards the degree of involvement of the feet.

2. *Intramuscular inoculation.* Only 10 cattle have been inoculated by the intramuscular route, 3 with strain MP, 2 with MPB, 1 with MC, 2 with MSL, and 2 with M 1, and all of these reacted with a severe generalized foot-and-mouth disease infection.

3. *Titration of Mexican strains of virus in cattle.* Observations have been limited so far mainly to the MP and M.1 strains of virus and it has been found from the accumulated results of titrations of suspensions of epithelium from tongue lesions that the mean 50% end-point for Mexican strains of virus, $10^{-6.1}$ (414 observations) for strain MP and $10^{-6.2}$ (394 observations) for strain M.1, is not appreciably different from that recorded for other strains of the virus of foot-and-mouth disease, *e.g.,* $10^{-6.8}$ for stock cattle strain 119 of the Vallée A type.

However, it has been noted that a number of titrations of Mexican strains, *e.g.,* MP and M.1, have been noteworthy for the difference in reactions between cattle in pairs used for this purpose. It seemed that the effect of the virus was impeded to a greater extent by any increased inherent resistance of the inoculated animal than was the case with other strains of the virus which have been studied. This would appear to be a more likely explanation than that the cattle used in these titrations were exhibiting greater variation in susceptibility than when other virus strains have been examined. It is interesting that although the mean 50% end-point of titrations of virus suspensions prepared from tongue lesions was not widely different from that observed with other strains of virus, yet the mean 50% end-point of titrations of defibrinated blood collected from cattle at the height of infection was somewhat lower, $10^{-2.4}$ (120 observations) for strain MP and $10^{-2.9}$ (60 observations) for strain M.1 as compared with $10^{-3.5}$ for our stock strain 119. Incidentally although an impression was received that individual comparative titrations of strains MP and M.1 showed in some cases a higher titer for the M.1 as compared with the MP strain, the accumulated results of all observations did not confirm this impression. The generally lower titer of the blood of cattle infected with Mexican strains as compared with that of animals infected with other strains of virus seems to fit into the picture of the lower invasiveness of Mexican virus strains examined. This lesser invasiveness was demonstrated in the first instance by a tendency to slower generalization after intradermal tongue inoculation but it is apparent to a greater degree in the experiments about to be described.

4. *Exposure of cattle to infection by contact.* As will be seen from Fig. 1, of 48 untreated susceptible cattle exposed in different experiments to virus infection with Mexican strains by contact with cattle showing well developed lesions, 20 failed to show any reactions. All the 48 cattle had been placed in contact with the virus donors from the time the latter were infected by intradermal inoculation of the tongue with a strong dose of virus, and had been left with them for a period of about 3 weeks. The important point is that they were with the virus donors throughout the whole of the acute stage of the infection.

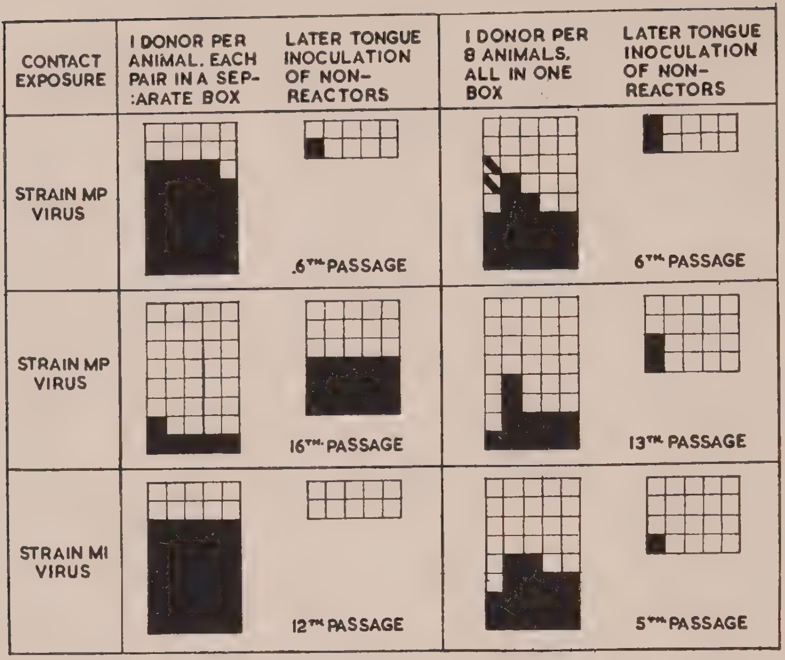
CONTACT EXPOSURE
I DONOR PER ANIMAL. EACH PAIR IN A SEP-:ARATE BOX
LATER TONGUE INOCULATION OF NON-REACTORS
I DONOR PER 8 ANIMALS. ALL IN ONE BOX
LATER TONGUE INOCULATION OF NON-REACTORS
STRAIN MP VIRUS
6TH PASSAGE
6TH PASSAGE
STRAIN MP VIRUS
16TH PASSAGE
13TH PASSAGE
STRAIN MI VIRUS
12TH PASSAGE
5TH PASSAGE

FIG. 1.

Contact exposure of susceptible cattle to Mexican strain infection. Each large rectangle depicts a group of cattle with a horizontal line of five squares for each animal. The first square represents the tongue and/or lips and the next four squares the four feet. A lesion at any particular site is denoted by blackening the appropriate square. A diagonal stripe is used when lesions occurred only on the lips and not on the tongue.

When these 20 non-reactors were inoculated subsequently into the tongue with at least 10,000 ID_{50} doses of homologous virus, 17 were shown to have developed a high grade of immunity, 11 having no reactions and 6 having reactions only at the sites of inoculation. The other 3 behaved in this severe test of resistance like unexposed fresh control cattle. It is to be noted that such development of an "inapparent infection" accompanied by a high grade of resistance to reinfection characterizes the foot-and-mouth disease pathological picture to a greater degree than is appreciated. In the writers' experience the phenomenon is much more pronounced in the case of the Mexican strains of virus under discussion than when contact exposure tests are made with other strains of virus.

In marked contrast observations may be cited on similar contact exposure tests with Pirbright cattle in which strain 119, the stock cattle strain of the Vallée A type was investigated.

Of 72 untreated susceptible control cattle (groups of 8 in 9 different experiments) placed in contact separately in boxes with single virus donors infected with strain 119 only 2 failed to develop visible lesions. These two were proved to have contracted an "inapparent infection" by the fact that they resisted the subsequent inoculation of 10,000 ID_{50} doses of the homologous virus. Again of 52 cattle (5 different experiments, four groups of 8 and one of 20) exposed to infection by contact with one virus donor, all developed a severe generalized infection.

It would appear to be justifiable to refer to the Mexican strains of virus as strains of reasonably good spreading power but of low invasiveness and strain 119 as one of good spreading power and high invasiveness.

Our experiments with other strains of virus have shown that some strains, *e.g.*, 39 and 336, may be intermediate in their invasiveness. This was evidenced in experiments in which susceptible cattle are exposed to infection for a short time or individually to single virus donors. They differ from the Mexican strains in that when groups of susceptible cattle are exposed to an accumulation of infection in one large box then they have all developed a severe generalized disease. An impression has been gained also that while certain strains of virus may exhibit lability in their spreading power and invasiveness from time to time others have greater stability in this respect. There is as yet no reliable information available on what factors, *e.g.*, method of storage, method of passage in animals, etc., are likely to affect the invasiveness of a strain. One of the difficulties lies in the setting up of an adequate organization for studies of this sort, and many strains may have been manipulated in the laboratory in one way or other to a considerable degree before being submitted to investigation. It is certain however that some strains, *e.g.*, 119 and the WA strain (a Vallée O strain which was recovered from cattle during the epizootic which spread rapidly across Europe during the period 1937-40), maintain their spreading power and invasiveness even after repeated passage in cattle by intradermal tongue inoculation or after storage in 50% glycerine for periods of from 4 to 7 years.

An attempt was made to enhance the invasiveness of the Mexican strain MP by infection of cattle by contact exposure passages but the series was broken by some of the cattle failing to develop lesions. Another attempt was made by rapid passages in cattle by intradermal tongue inoculations (6th to 13th passage) but as will be seen from Fig. 1 the invasiveness was not increased in the tests made after the 13th and 16th passages.

Let it be mentioned also that strain M.1 recovered from cattle in a field outbreak in Mexico in October, 1947, when the disease had been spreading for about a year exhibited the same low invasiveness as strain MP recovered from cattle in Mexico in December, 1946.

5. *Intranasal instillation of virus.* It is noteworthy that this low invasiveness of strain 39 (*vide supra*) and the more pronounced low invasiveness of the Mexican strains MP and M.1 is exhibited also when single or repeated large doses of highly active virus are administered by intranasal spray and inapparent infections may develop in the same way under such conditions of experiment.

The result of one experiment with the Mexican virus strain M.1 may be cited. Four susceptible cattle received 3 doses of a gradocol membrane (Elford) virus filtrate with a titer of 10^{-4} at 24 hours interval. One animal developed lesions on the 5th day followed by a severe generalized infection involving the mouth and all four feet by the 6th day. The three others remained free of lesions and after a fortnight they were inoculated intradermally into the tongue with a filtrate of a titer of 10^{-4}. Two showed no lesions and the third had local lesions at the site of inoculation only which regressed rapidly.

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Strains of Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Complement Fixation Tests.

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On the basis of cross-immunity tests, the great majority of strains of the virus of foot-and-mouth disease can be classified as belonging to one of 3 immunological types, known as Vallée O, Vallée A, and Waldmann C. It has been suspected, however, almost since the types themselves were established, that differences of a lesser degree existed in the antigenic behavior of some strains within one immunological type. Such suspicion arose from observations on cross-immunity in cattle and guinea pigs, but owing to the difficulties associated with experiments in large animals with this highly infective virus, and the deficiencies of experiments in laboratory animals, arising from possible alterations in strains during passage, precise observations are lacking. The differences between such strains within a type in any case appeared to be of small significance as compared with the very clear-cut demarcation of the types themselves.

When work was proceeding at this Institute, during 1947, on strains of virus recovered from the present epizootic in Mexico, this phenomenon of "variant" strains was encountered in a new and important connection. It was found that, although a particular strain (MP), from Mexico, could be classified as of Vallée A type, vaccination of cattle with a potent vaccine prepared from the Pirbright stock cattle strain 119 of the same type, gave poor protection against the Mexican strain. This result is reported in detail elsewhere.[1]

To obtain further information on this discrepancy, opportunity was taken to apply one of the more recent methods of quantitative study of the virus and its antibody. Cross-neutralization tests were carried out as described in a subsequent paper of this series, and the results confirmed the existence of an antigenic difference between strains MP and 119.

While this work was in progress, our attention was drawn to papers by Traub and Rodriguez[2] and Traub and Möhlmann.[3] The authors had, in the course of examination of field strains by the complement fixation method encountered 2 classes of "variant" strains of the virus, one of them being within the Vallée A type group. Some of these strains were recovered from vaccinated cattle which had later developed the disease. They could be shown to be different in their complement fixing properties from the stock vaccine strain of Vallée A type by cross tests with guinea pig antisera prepared against the individual strains. Traub and Möhlmann drew attention to the complications introduced into vaccination against the disease by the occurrence of these variant strains and it is unfortunate that the war has prevented them from publishing in full their protocols.

The development of complement fixation as a method of "typing" the virus of foot-and-mouth disease dates from the observations of Ciuca[4] in which guinea pig antigen and antiserum were used. Traub and Möhlmann[5] produced reliable and consistent results with bovine virus as antigen, an advance which increased greatly the usefulness of the test. Rodriguez, Prado, and Palacios,[6] working independently, also showed the value of the method. Work at this Institute has been directed at increasing the precision of measurement of the reaction, using methods already described.[7] Application of these methods to the Mexican strains, together with the use of strictly homologous antisera, soon confirmed the existence of differences between them and strain 119, the stock Vallée A strain. The data on these observations are presented below.

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[1] Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 77.

[2] Traub, E., and Rodriguez, R., *Zbl. Bakt. Abt.*, Orig., 1944, **151**, 380.

[3] Traub, E., and Möhlmann, H., *Berl. und Münch. tierärztl. Wschr.*, 1946, **1**, 1.

[4] Ciuca, A., *J. Hyg. Camb.*, 1929, **28**, 325.

[5] Traub, E., and Möhlmann, H., *Zbl. Bakt. Abt.*, Orig., 1943 **150**, 289, 300.

Methods. The technic used follows that described in the differentiation of vesicular stomatitis and foot-and-mouth disease,[7] and the results are presented in the same way, all end-points being referred to the dose of complement necessary for 50% hemolysis. For the differentiation of variants, it is essential to prepare a strain-homologous antiserum. So far, no success has attended experiments with bovine antisera and all results were obtained with antisera prepared against the guinea pig adapted strains.

Serum from convalescent guinea pigs was used in one or two cases but in the remainder a course of intramuscular inoculations with virus of the strains concerned was given to the convalescent guinea pigs. The antigens were 1:4 or 1:10 suspensions of infective bovine tongue epithelium, ground in M/25 phosphate buffer solution with sand. They were prepared from 4-24 hours before use and centrifuged for 20 minutes at 2,000-3,000 r.p.m. Complement and the hemolytic system were the same as in the earlier experiments.[7] The strains used were:

TABLE I.
Complement Fixation with Vallée A Type Variants.

Antiserum	Virus strain			
	119	MP	M.1	Nil
119a	.76	.62	.62	.53
119b	.80	.61	.62	.53
MP	.51	.69	.56	.53
M.1	.52	.49	.79	.53
Nil	.50	.39	.36	.53

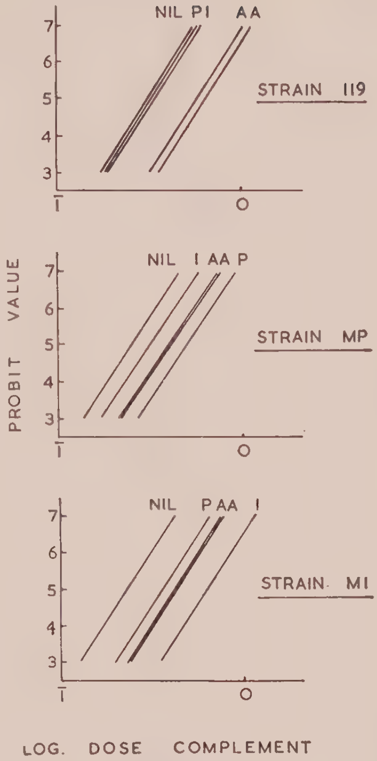

FIG. 1.
Complement-fixation with Vallée A Type Variants. The antisera used are indicated thus: A, A—Two batches of strain 119 antiserum. P—Strain MP antiserum. I—Strain M.1 antiserum.

Vallée O Type. Strain 1. A strain from cattle recovered from a field outbreak in 1924 and since maintained in guinea pigs.

Strain 39. A strain from cattle recovered from a field outbreak in 1927 and since passaged in cattle.

Vallée A Type. Strain GB. A guinea pig adapted strain received from the island of Riems, Germany, in 1929.

Strain 119. A strain from cattle recovered from a field outbreak in 1932 and since passaged in cattle.

Strain MP. A strain from cattle recovered in Mexico, December, 1946.

Strains M.1 and M.2. Strains from cattle recovered in Mexico, October, 1947.

Strain AX. A cattle-passaged strain received from the Argentine in 1944.

Waldmann C Type. Strain GC. A guinea

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[6] Rodriguez, R., Prado, M., and Palacios, R., *Bol. Soc. Paul. Med. vet.*, 1945, **7**, 94.

[7] Brooksby, J. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 254.

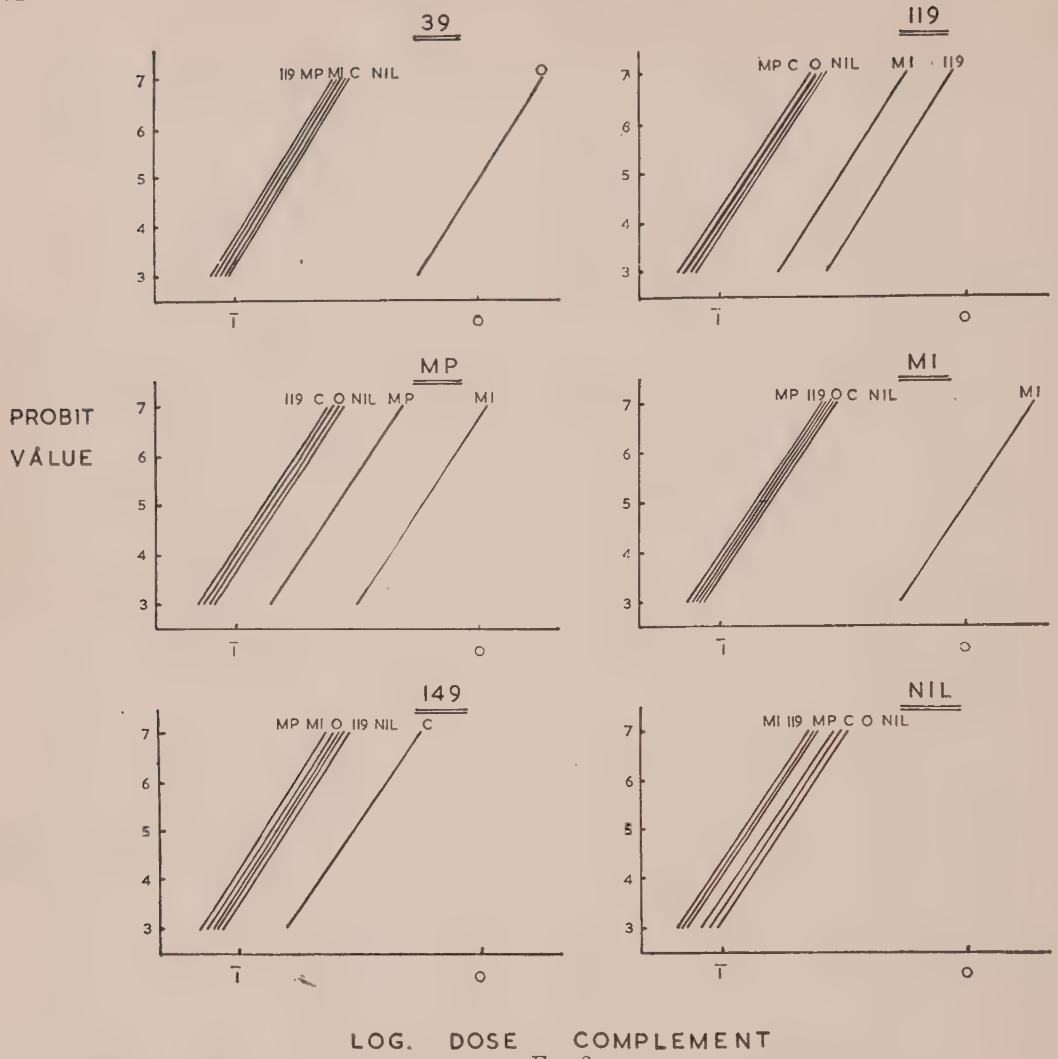

FIG. 2.

The relationship of Vallée A type variants to other strains. The antisera are indicated thus: O—Strain 1 antiserum, Vallée O type. 119—Strain 119 antiserum, Vallée A type. MP—Strain MP antiserum, Vallée A type. M.1—Strain M.1 antiserum, Vallée A type. C—Strain GC antiserum, Waldmann C type.

pig adapted strain received from the island of Riems, Germany, in 1933.

Strain 149. A strain from cattle recovered from a field outbreak in 1934 and since passaged in cattle.

Results. The result of a simple experiment illustrating the detection of variants is presented in Table I and shown graphically in Fig. 1. Three antigens, from strains 119, MP and M.1, prepared as 1:10 suspensions of infective bovine epithelium, were tested against antisera from guinea pigs convalescent from infection with these strains. Two samples of strain 119 antiserum were included. They behaved consistently. In the table, the values are the logarithmic doses of complement corresponding to the 50% hemolysis end-points in the presence of the various antigen-antiserum mixtures. The effect of the various sera on complement has been allowed for by applying a correction to all the figures in each horizontal row in the table so that the serum control series (without antigen) give the same value. From the

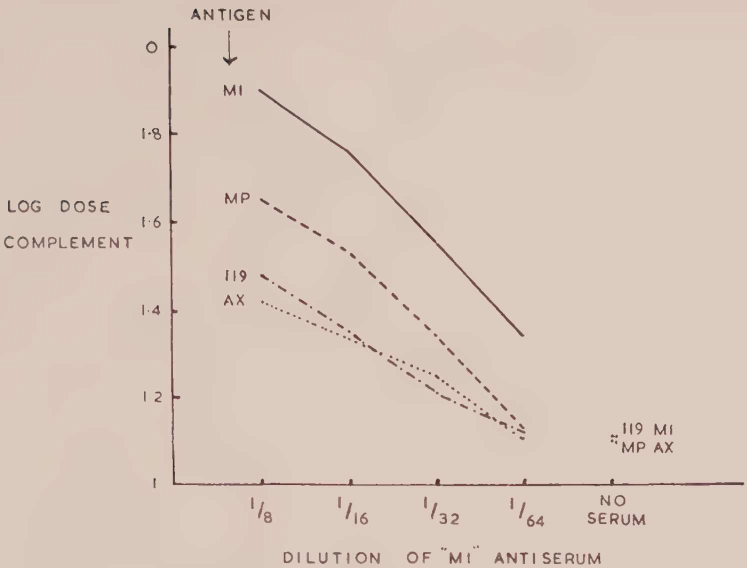

Fig. 3.
The effect of dilution of antiserum on cross-fixation with Vallée A type variants.

graphs in Fig. 1, it is clear that the homologous mixtures show greater fixation in each case, or, as is equally important, the relative efficacy of fixation by the various antisera depends on the antigen present. Fixation is indicated by the line concerned being further to the right.

Further observations on the relation of the variants to each other and to the immunological types are presented in Fig. 2. The result is shown of tests of strains 39, 119, MP, M.1, and 149 with the guinea pig antisera against strains 1, 119, MP, M.1, and GC. In the case of strains 39 and 149 the result is clear cut in that no serum other than the homologous shows any fixation. In the Vallée A group, strain 119 virus shows fixation, better with 119 antiserum but also with M.1 antiserum; MP virus fixes best with M.1 antiserum but also with MP antiserum, and M.1 virus shows very strong fixation with M.1 antiserum. This result would suggest that M.1 might be a "universal" A antiserum; but it is more likely to be due to a high concentration of antibody. The MP antiserum was from convalescent guinea pigs, as opposed to the other sera involved which were obtained from hyperimmunized guinea pigs. The result with MP antigen was therefore, in all probability an expression of the low titer of the MP antiserum and high titer of the M.1 antiserum. Such anomalous effects could be counteracted by titration and suitable dilution of the serum before use in the test. This view is supported by the result shown in Fig. 3, where dilutions of the same M.1 antiserum were prepared and tested against the strain homologous virus and 3 other Vallée A antigens, 119, MP, and AX. The log. doses of complement giving 50% hemolysis are plotted against the dilutions of serum and a curve obtained by joining the points for each antigen. It is apparent from this graph that if a 1/64 dilution of serum were used in this test, fixation would be shown with only the homologous strain, the reaction with other strains of the same type having disappeared. This finding agrees well with that shown in Traub and Möhlmann's paper relating to the variants within the Vallée A immunological groups encountered by them.

In carrying out the routine type tests, the stock antisera used are from hyperimmunized guinea pigs. The aim here is to ensure fixation when testing with any strain of the same type, and for this purpose a high titer is an advantage. A test of the several variant strains of Vallée A type with the stock GB antiserum did, in fact, give the result that all the strains fixed complement in the presence of GB antiserum, although there were at least 3 distinct variants in the group of strains used.

Discussion. The occurrence of variant strains has imposed new conditions on the successful application of the complement fixation test in foot-and-mouth disease, as well as emphasizing the importance of precision in carrying out the test. In the light of the results reported here the necessity for standardization of reagents is clear. Definition of units for antiserum and antigen must await more extended observations. The other essential would appear to be the use of a wider range of antisera, including in most cases, one which is strain homologous. As mentioned earlier, only further work will show what differences in respect of complement fixation must be demonstrated before the differences between strains are of practical moment in immunization.

Summary. Two strains of the virus of foot-and-mouth disease, of Vallée A type, have been found to differ antigenically from each other and from the stock strain of this type used in this Institute.

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Strains of Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Serum Neutralization Tests.

J. B. BROOKSBY, IAN A. GALLOWAY, AND W. M. HENDERSON.
(Introduced by R. E. Shope.)
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The neutralization of the virus of foot-and-mouth disease by specific antisera has not been studied extensively. Bedson, Maitland and Burbury[1] described a technic in which guinea-pigs were used as test animals. They referred to the absence of cross neutralization between virus and antiserum of different immunological types. In examining 2 strains of the same type, they observed, in one experiment, that although the antiserum prepared against one strain neutralized to some extent virus of both strains, there was greater neutralization in the homologous mixtures. The errors implicit in their test, however, were great enough to make the validity of their conclusions somewhat doubtful. The development of a neutralization test in cattle using the method of inoculation of multiple sites on the tongues of cattle has the advantage of greater accuracy and also makes it possible to deal with both antiserum and virus from the naturally susceptible species. Preliminary investigations suggested that this method might be used for the identification of immunological types of virus in cattle and for the detection of even finer antigenic differences. Again, as in the use of the complement-fixation test in this sphere, caution must be applied to the interpretation of results and transference of conclusions to the field of vaccination experiments. Only more extensive studies can establish the extent of the correlations to be expected between this and the other methods of measurement of antigenic differences.

Methods. The strains of virus used have been described.[2,3] The antisera were from cattle convalescent from 14 to 21 days after the earliest lesion of the disease had appeared. Pooled samples of serum from 4 or more animals were used. The details of technique followed, in general, those described earlier.[4] In all but one of the observations, mixtures of diluted virus and excess bovine serum were prepared, so that, for example one volume of a 10^{-2} dilution of virus filtrate was added to 9 volumes of the antiserum concerned, giving a 10^{-3} dilution in antiserum.

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[1] Bedson, S. P., Maitland, H. B., and Burbury, Y. M., Foot-and-Mouth Disease Research Committee, 2nd Progress Report, 1927, London, p. 95.

[2] Brooksby, J. B., Henderson, W. M., and Galloway, Ian A., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 64.

[3] Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 70.

From 30-90 minutes after preparing the dilutions of virus in antiserum, the series were inoculated into the tongues of cattle. Four groups of 5 sites were inoculated in one animal, each group with one serial dilution. Two animals were used for each series so that 10 observations were available on each serial dilution. The calculation of the 50% end-point dilution is therefore justified. Inoculations were made when the animal was narcotized with Pentothal Sodium (Abbott),[5] and observations on the occurrence of lesions at the sites inoculated were made at approximately 20 and 28 hours after inoculation.

The amount of virus neutralized was arrived at by deducting the titre (*e.g.* $10^{-2.5}$) of the virus in the presence of antiserum from the titre (*e.g.* $10^{-5.2}$) of the virus diluted in Hartley's Broth, giving for this example $10^{-2.7}$. The results of comparative titrations of virus in normal serum showed that little error could be attributed to non-specific effects.

An alternative method was used in one experiment described below. Dilutions of antiserum were prepared and each was mixed with an equal volume of a virus filtrate suitably diluted, as estimated on the basis of an earlier titration. In this case the dilution of antiserum corresponding to the 50% end-point was calculated from the result.

Results. The results of 4 experiments are represented diagrammatically in Fig. 1-4. On Fig. 1-3 are presented the data on the interrelationship between the Pirbright stock strain of Vallée A type, 119, and the 2 Mexican strains MP and M.1. It will be noted that for the comparison of strains 119 and MP, the observations for both serum-dilution and virus dilution methods are given. In both cases the neutralizing powers of the antisera were greatest against the corresponding strains.

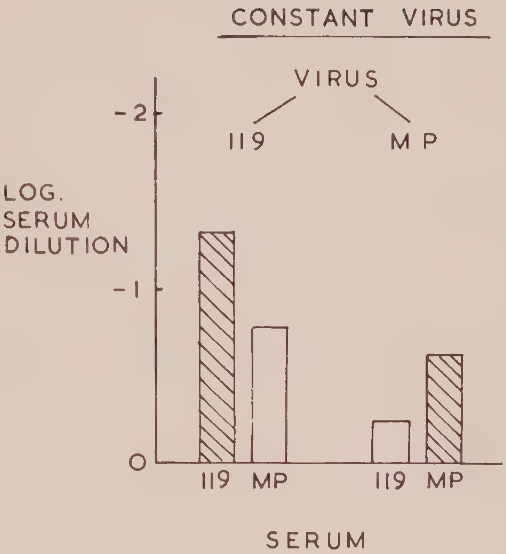

FIG. 1.
Neutralization test with viruses and antisera of two Vallée A type strains.

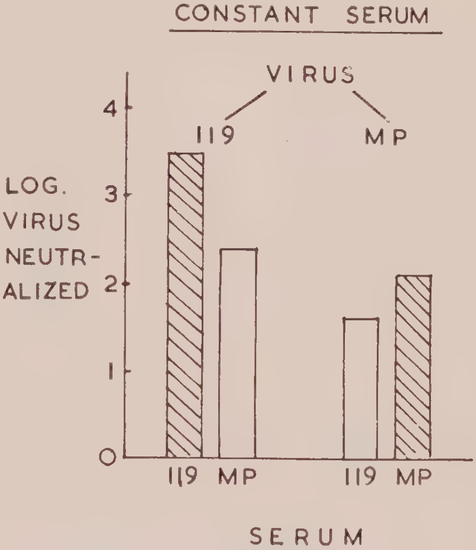

FIG. 2.
Neutralization test with viruses and antisera of two Vallée A type strains.

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[4] Brooksby, J. B., 1946, Thesis, University of London, (in press as an Agricultural Research Council Report.)

[5] Henderson, W. M., *J. Comp. Path.*, 1944, **54**, 245.

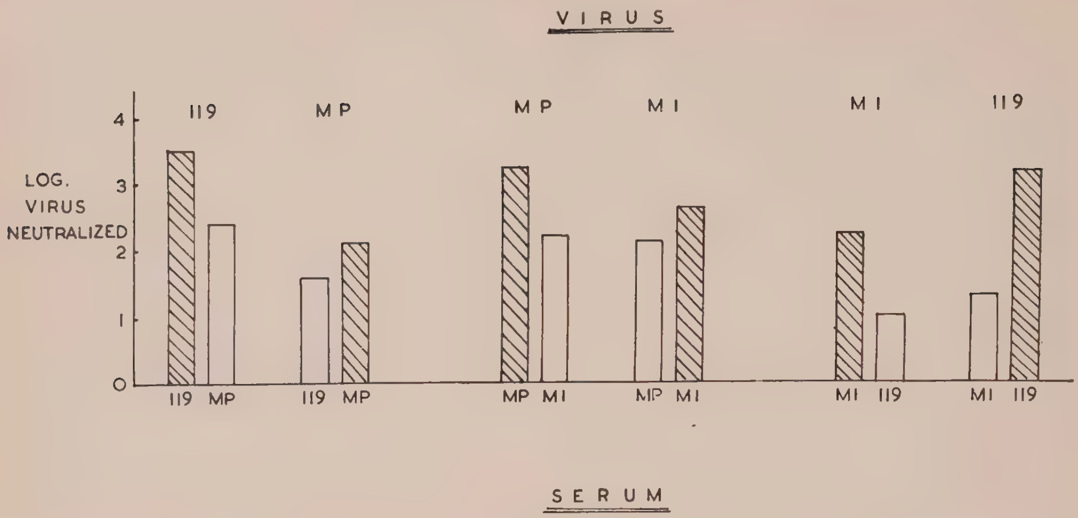

FIG. 3.
Neutralization tests with viruses and antisera of three Vallée A type strains.

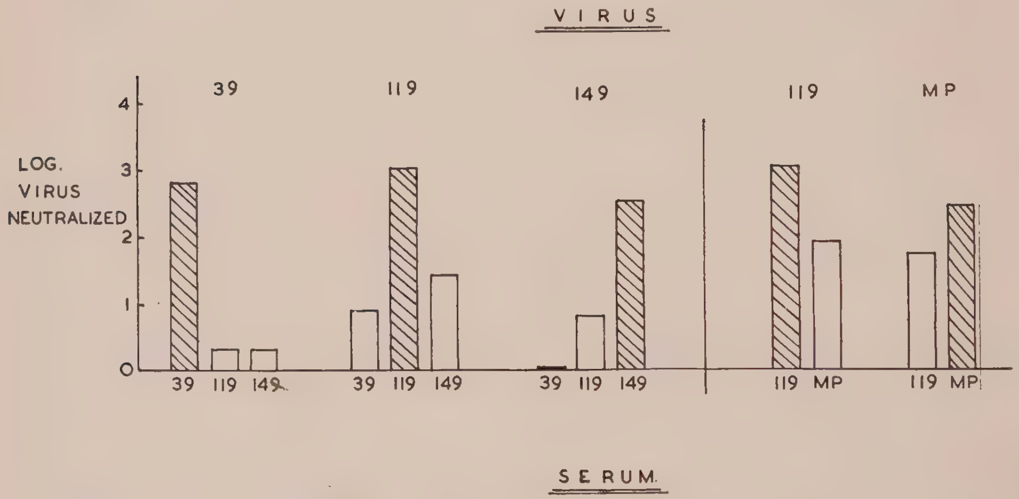

FIG. 4.
Neutralization tests with viruses and antisera of strain 39 (Vallée O type) strains 119 and MP (Vallée A type) and strain 149 (Waldmann C type).

A useful expression of the difference in relative activity of two antisera is given by the sum of the logarithmic differences in the amounts of virus neutralized by them when they are tested against first the strain corresponding to one of them and secondly the strain corresponding to the other. Thus in Fig. 2 the logarithmic difference between the amount of virus of strain 119 neutralized by strain 119 and strain MP antisera was 1.1, while for virus of strain MP, the logarithmic difference was 0.5. The sum of logarithmic differences for these 2 antisera is therefore 1.6, and this value may be called the "differential neutralization" as between strains 119 and MP. From Fig. 3, similar calculation gives the value of the differential neutralization between strains MP and M.1 as 1.5 and between strains M.1 and 119 as 3.0.

The high figure obtained with strains M.1 and 119 led to an investigation of the differences between strains of different immunological type. The results of this experiment are represented in Fig. 4, which also repeats the observations on strains 119 and MP. The figures for differential neutralization derived from these results are

Between strains 39 and 119 (Vallée O and A types)	4.6
Between strains 39 and 149 (Vallée O and Waldmann C types)	5.0
Between strains 119 and 149 (Vallée A and Waldmann C types)	3.3
Between strains 119 and MP	1.9

Thus only in the case of strains 119 and M.1 does the magnitude of a strain difference nearly equal a type difference.

Discussion. The antibody produced in response to infection appears to differ qualitatively from strain to strain of those examined. The importance of these differences in immunization may depend on a number of factors. The antibody response of an animal to an injected vaccine may not reproduce qualitatively the response to an infection even if the preparation of vaccine from a particular strain has preserved the antigenic peculiarities of that strain. Again the quantitative factors influencing immunization may render strain specificity less important than it would at first appear. Further comment must be deferred until observations have been accumulated on these aspects. Strains shown by cross-immunity tests in vaccination experiments to be more closely related than those mentioned in this paper require to be examined, and work on these lines is continuing.

Summary. (1) Differences between strains of the virus of foot-and-mouth disease of one type have been demonstrated by cross-neutralization tests in cattle. (2) The magnitude of such differences in these experiments is not so great as that between immunological types.

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Strains of the Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Vaccination Experiments.

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(Introduced by R. E. Shope.)
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Experiments on the immunization of cattle against infection with strains of virus from Mexico were made to determine whether vaccines prepared from the highly immunogenic Pirbright stock cattle strain 119 of the Vallée A type would protect them against infection with these strains which are of the same type. The results of these early experiments showed a difference in the antigenic behavior of strain 119 and the Mexican strain MP. This observation led to a study of these and other Mexican strains by methods described elsewhere.[1,2] Observations were made also on the immunizing value of vaccines prepared from the Mexican strains themselves. The problem became complicated later by failure of these Mexican strains to infect susceptible cattle, in control groups included in contact infection tests, with the regularity associated with highly invasive strains.

The pattern of the experiments was the same in each case. Vaccines were prepared from different strains, groups of cattle were injected with these vaccines and 2 to 3 weeks later, the protection established was tested with the same strain or another strain of the same immunological type.

Methods. Particulars of the strains of virus

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[1] Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 70.

[2] Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 74.

MP, M.1 and 119 used in these experiments have already been given.[1,3]

Preparation of Vaccines. Vaccines prepared by the inactivation of infective blood were chosen for these experiments. The use of this type of vaccine as a prophylactic in foot-and-mouth disease was reported first by Graub, Zschokke and Saxer[4] and the general principles of its preparation have since been followed at this Institute in developing inactivation tests, vaccine potency tests and for studying the difference in antigenicity of strains of virus and in other immunological problems.

Four or five litres of blood were collected from the jugular vein of each of a group of 2, 3 or 4 cattle that had been inoculated on the tongue with freshly passaged virus material.

The cattle were bled between the 24th and 48th hour after inoculation at a time when secondary lesions were developing. The blood was defibrinated, the samples pooled and a 1% solution of Du Pont "extra pure" crystal violet added to give a final concentration of 0.05%. The inactivation treatment consisted of 6 or 8 days incubation at 37°C. The titre of the pooled blood was determined by our routine method using cattle.[5] Vaccines prepared from each new strain studied were tested for inactivation of the virus after the requisite period of incubation at 37°C. The criterion for inactivation or non-infectiveness was the recording of consistently negative results in 80 to 120 observations made on the sites of multiple tongue inoculations of a group of 8 to 12 cattle.

Vaccination of cattle. Groups of Devon steers, 1½ to 2 years old were vaccinated by subcutaneous injection into the dewlap in the region of the brisket. The vaccines were usually stored at 4°C for a short time after completion of incubation at 37°C. In no case was this period longer than 23 days. In other experiments the potency of this type of vaccine has been proved to remain unaltered for at least 4 months at either 4°C or frozen at –20°C.

Exposure of Vaccinated and Control Cattle to Infection by Contact. The cattle were housed in loose boxes 12 feet square and in contact exposure tests one vaccinated or unvaccinated control animal was left in a box with an infected animal for at least 14 days. This virus donor was a susceptible animal which was inoculated in the tongue with virus at the time the 2 animals were placed together.

Tongue Inoculation of Vaccinated and Control Cattle. The inoculum in each case was a 0.55μ gradocol (Elford) membrane filtrate of the supernatant fluid of a centrifuged suspension of freshly collected vesicle epithelium. The filtrate was titrated in cattle and a dilution made to provide an inoculum of the desired strength. Each animal was inoculated intradermally on the tongue at 10 separate sites using 0.1 cc at each site. The result of a titration is based on similar inoculations of 0.1 cc amounts. Thus if the 50% positive end-point of the inoculum was 10^{-4} then each of the 10 sites on the vaccinated or control animals' tongue would receive approximately 10,000 50% end-point infective doses *i.e.* 10,000 I.D. 50.

Examination of Animals. The mouth and feet of the cattle were examined daily, at first, and later on every second or third day for 3 weeks after contact exposure and for 2 weeks after inoculation.

Results. Experiment A. Contact exposure test with strains 119 and MP vaccines and viruses. Strain 119 Vaccine No. 93—Titre of blood $10^{-3.6}$.

Strain MP Vaccine No. 94—Titre of blood $10^{-3.2}$.

The results of previous experiments (as yet unpublished) had shown that from 6 of 8 to 8 of 8 cattle which had received a 30 cc dose of a strain 119 vaccine resisted infection by contact with virus donors inoculated respectively with Vallée A type strains 119, A. Cor.

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[3] Brooksby, J. B., Henderson, W. M., and Galloway, Ian A., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 64.

[4] Gräub, E., Zschokke, W., and Saxer, E., *Schweiz. Arch. Tierheilk.*, 1939, **81**, 436.

[5] Henderson, W. M., 1945, Thesis, University of Edinburgh, (in press as an Agricultural Research Council Report).

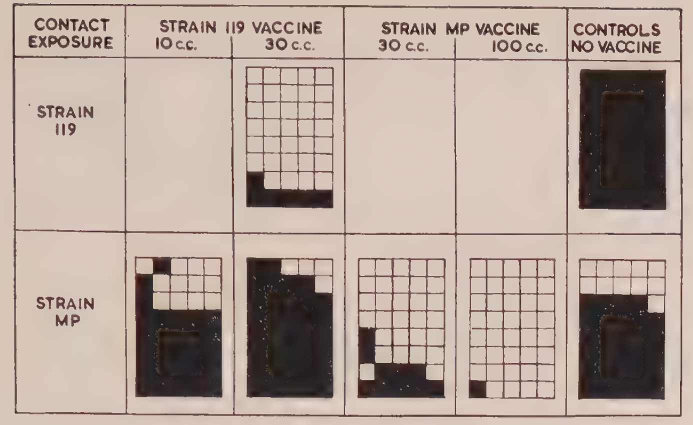

FIG. 1.
Experiment A. Contact exposure test with strains 119 and MP vaccines and viruses. In this and subsequent figures each large rectangle depicts a group of cattle with a horizontal line of five squares for each animal. The first square represents the tongue and/or lips and the next four squares the four feet. A lesion at any particular site is denoted by blackening the appropriate square.

and AX. In the present experiment, 2 groups of cattle injected respectively with 10 cc and 30 cc doses of vaccine No. 93 (strain 119), were exposed to infection by contact with virus donors inoculated with strain MP virus. As a control of the potency of the vaccine another group injected with 30 cc were exposed in a similar way to infection with strain 119. Vaccine No. 94 (MP) was tested by exposing two groups of cattle injected respectively with 100 cc and 30 cc doses to infection with strain MP virus. The results of the experiment represented diagrammatically in Fig. 1 show that although vaccine 93 (strain 119) gave the expected degree of protection against the 119 strain, an equivalent amount of vaccine did not protect any of 8 cattle against infection with strain MP. A clinically recognizable disease did not develop in all of the control group of susceptible cattle exposed to infection by contact under similar conditions and this point is discussed further when the results of the next experiment are dealt with. The results show also that the protection conferred on cattle by the strain MP vaccine against infection with strain MP was not of such a high order as that given by a strain 119 vaccine against infection with strain 119.

Experiment B. Contact exposure test with strains 119 and MP vaccines and viruses.

Strain 119 vaccine No. 96—Titre of blood $10^{-3.7}$.

Strain MP vaccine No. 95—Titre of blood $10^{1.5}$.

It has already been mentioned[6] that the titre of the blood of our cattle infected with Mexican strains tended to be somewhat low. As the titration of infective material only assesses the amount of active virus, a low titre might not necessarily indicate the true antigenic level. It was for this reason that strain MP vaccine No. 95 was used in this experiment even although the titre of the blood was much lower than our usual standard for vaccine preparation.

The experiment was similar to experiment A. A dose of 100 cc of the strain 119 vaccine was injected into one group of cattle to determine whether increased dosage would compensate for the apparent antigenic dissimilarity between strains 119 and MP in exposure to infection against strain MP and another group received 30 cc doses as a link with the previous experiment.

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[6] Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 66.

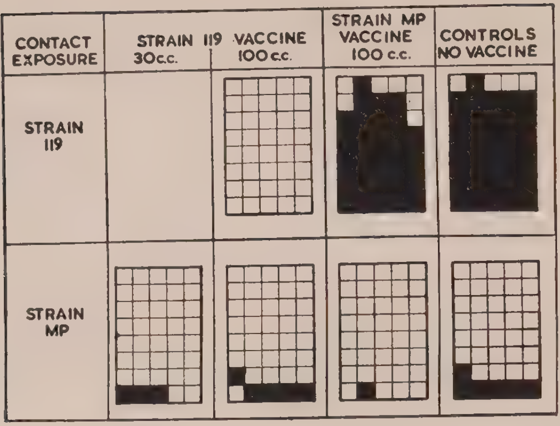

Fig. 2.
Experiment B. Contact exposure test with strains 119 and MP vaccines and viruses.

The strain MP vaccine was tested against infection with strain 119, as well as against infection with strain MP.

The results are shown in Fig. 2. The failure of 6 of the 8 cattle in the unvaccinated MP control group to react to infection renders negative results valueless in interpreting that part of the experiment involving exposure to infection with strain MP. The facts that 6 out of 8 controls as against the 2 out of 8 of the last experiment failed to react to exposure to infection and that 3 of the 6 non-reactors when subsequently tested for susceptibility by tongue inoculation reacted like fresh susceptible cattle suggests a change in the behavior of strain MP from experiment to experiment. This is perhaps associated with a variation in the invasiveness of the strain. The altered conditions in the present experiment no doubt account for the fact that only 1 of 8 as against 8 out of 8 cattle in *experiment A*, injected with a 30 cc dose of strain 119 vaccine reacted when exposed to infection with strain MP.

The results of the exposure of vaccinated cattle to infection with strain 119 showed that the protective value of the strain 119 vaccine was up to expectation. The complete failure of the strain MP vaccine in a dose of 100 cc to protect cattle against infection with strain 119 is unfortunately impossible to interpret as the other half of the experiment provided no satisfactory estimate of its potency against infection with the strain homologous virus MP. More extended observations are necessary to determine whether this lack of protection of cattle by the strain MP vaccine, under discussion, against infection with strain heterologous virus 119 could be attributed to the low titre of the blood used in preparing the vaccine or was a real antigenic dissimilarity.

Two facts had arisen during the course of this work. Firstly that clinical reactors to the Mexican strains of virus did not always yield blood of adequate titre for vaccine production. Secondly that these strains were unsuitable for use in contact infection experiments owing to their inability to produce visible signs of foot-and-mouth disease in all the cattle of an unvaccinated control group.

Accordingly, in the next experiment a vaccine was prepared by adding a filtrate to infective blood to ensure having starting material of good titre. Also it was decided that the method of testing for immunity would be altered to the intradermal inoculation of the tongue with a filtrate of known virus potency in the hope that this would be followed by complete reactions in the animals of the control groups. Sufficient data have not been accumulated yet to show how the results of the tongue inoculation of vaccinated cattle can be correlated with those of infection by contact exposure tests. A preliminary experiment, Fig. 3, with a highly in-

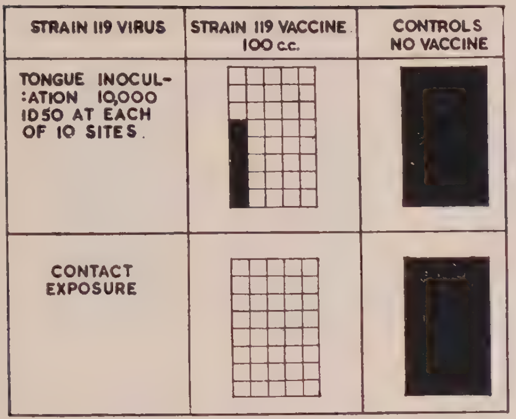
STRAIN 119 VIRUS
STRAIN 119 VACCINE 100 c.c.
CONTROLS NO VACCINE
TONGUE INOCUL-:ATION 10,000 ID50 AT EACH OF 10 SITES.
CONTACT EXPOSURE

FIG. 3.
Comparison of tongue inoculation with contact exposure of test animals in a vaccination experiment. Strain 119 vaccine and virus.

vasive strain, 119, showed that when the dose of vaccine injected is large, protection against development of secondary lesions in inoculated cattle corresponds to complete protection against contact infection.

Experiment C. Tongue inoculation and contact exposure test using strain M.1 vaccines and virus.

Strain M.1 vaccine No. 102—Titre of blood $10^{-2.8}$.

Strain M.1 vaccine No. 103—Titre of blood $10^{-2.8}$ with the addition of a filtrate of an epithelial suspension of tongue vesicular lesions to give a final titre of $10^{-3.7}$.

Two different inocula were used, one having a titre of $10^{-4.7}$ and the other a 1:1000 dilution of the first, having a titre of $10^{-1.7}$. The doses of the vaccines injected were 100 cc. The results are shown in Fig. 4. While the complete absence of secondary lesions in all the vaccinated groups demonstrates the high degree of protection that can be conferred on cattle by this type of vaccine prepared from this strain of virus, it did not permit the relative merits of the two vaccines to be assessed. Nor did it allow the demonstration of the advantage of giving 2 successive smaller doses of vaccine instead of one large one. This improved effect has been demonstrated in work (unpublished) with other strains of virus when the successive doses of vaccine were less than in the present instance.

It will be noted also that there was little difference in the effects produced respectively by the small or the large amounts of virus in the two inocula. It will be appreciated that once a primary lesion is produced the

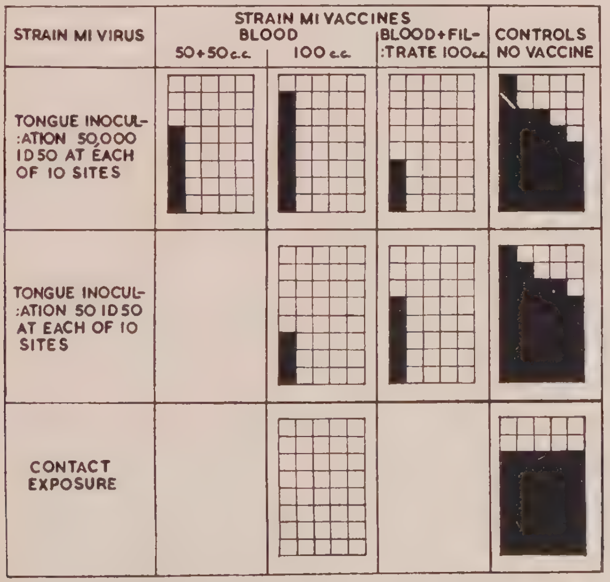
STRAIN MI VIRUS
STRAIN MI VACCINES
BLOOD
50+50 c.c.
100 c.c.
BLOOD+FIL-:TRATE 100 c.c.
CONTROLS NO VACCINE
TONGUE INOCUL-:ATION 50,000 ID50 AT EACH OF 10 SITES
TONGUE INOCUL-:ATION 50 ID50 AT EACH OF 10 SITES
CONTACT EXPOSURE

FIG. 4.
Experiment C. Tongue inoculation and contact exposure test with strain M.1 vaccines and virus. A white diagonal stripe denotes a secondary lesion on the lip following a primary lesion on the tongue.

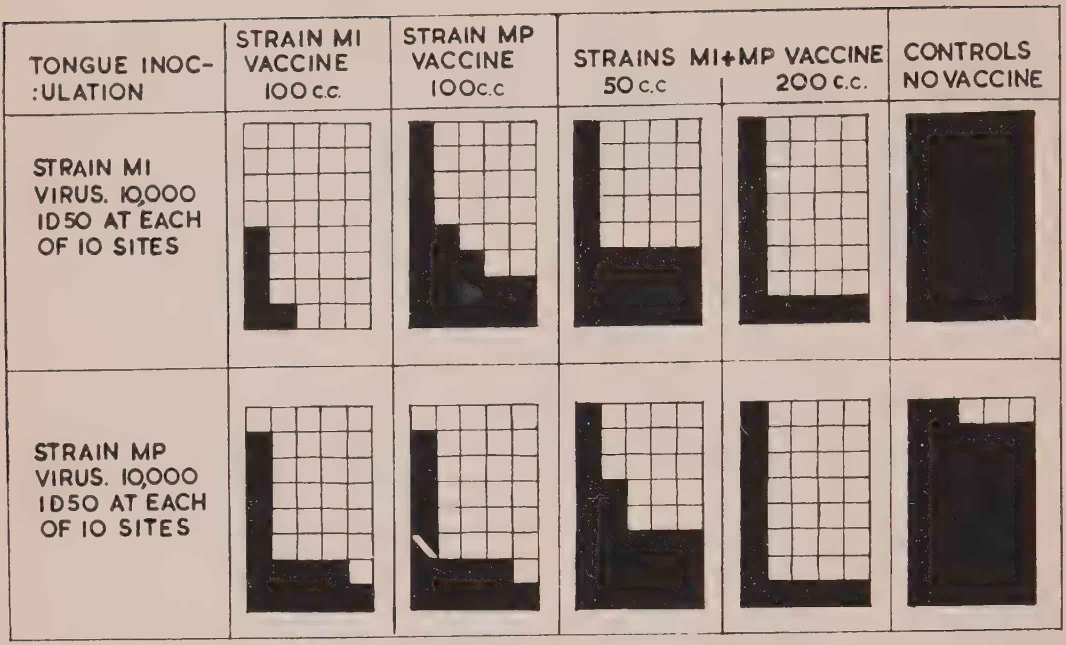
TONGUE INOC-:ULATION
STRAIN MI VACCINE 100 c.c.
STRAIN MP VACCINE 100c.c
STRAINS MI+MP VACCINE
50 c.c
200 c.c.
CONTROLS NO VACCINE
STRAIN MI VIRUS. 10,000 ID50 AT EACH OF 10 SITES
STRAIN MP VIRUS. 10,000 ID50 AT EACH OF 10 SITES

FIG. 5.

Experiment D. Tongue inoculation test with strains M.1 and MP vaccines, a "bivalent" vaccine incorporating strains M.1 and MP, and strains M.1 and MP viruses. A white diagonal stripe denotes a secondary lesion on the lip following a primary lesion on the tongue.

quantity of virus originally inoculated bears no relation to the severity of the test for the animals have then to contend with the greatly increased amount of virus released from the focus of multiplication. The non-appearance of a primary lesion in a vaccinated animal following the tongue inoculation of virus is undoubtedly of significance. Nevertheless the production of a primary lesion must constitute a severe test of the animal's immunity.

A contact exposure test was carried out at the same time and the cattle of the control groups of the tongue inoculation test were used as virus donors. Eight cattle injected with 100 cc of vaccine No. 102 were exposed to virus infection by contact and there was a group of 8 unvaccinated control cattle.

Although here again the incomplete reaction of the cattle of the control group complicates the picture to some extent, the result of the comparative test confirms that shown in Fig. 3 in the experiment in which strain 119 was used.

Experiment D. Tongue inoculation test using strain M.1 and MP vaccines, a "bivalent" vaccine incorporating strains M.1 and MP, and strain M.1 and MP viruses.

Strain M.1 vaccine No. 105—Titre of blood 10^{-3}.

Strain MP vaccine No. 106—Titre of blood $10^{-2.6}$.

Strain M.1 + MP vaccine No. 107—a mixture of equal quantities of vaccines Nos. 105 and 106 made at the completion of the incubation of each component.

This experiment was planned as the first of a series to determine the significance in the immunization picture of the differences recorded in the complement fixation and serum neutralization tests between strains M.1 and MP. The titres of the respective inocula of the 2 strains were approximately the same *viz.* 10^{-4}. Primary lesions were produced in the majority of the animals and the estimate of the efficacy of the vaccines was based largely on the non-development of secondary lesions. Although each vaccine gave better protection against the strain of virus used in its preparation than against the other, Fig. 5, the differences in the results between the groups were not of greater magnitude than to indicate more than a trend in the direction of some antigenic dissimilarity between strains M.1 and MP such as that strongly suggested by the results of their examination in complement fixation and serum neutralization tests. This dissimilarity may have been masked by the large dose of vaccine administered. Further observations are necessary in experiments in which a series of varying doses of vaccine will be used before full significance can be attributed to the results of the different methods of examination of strains of virus.

The results show that the lesser protection conferred by the strain MP vaccine is consistent with the lower titre of the blood used in its preparation. The "bivalent" vaccine incorporating both strains induced a comparable resistance against infection with either strain when the dose was adequate.

Summary and Conclusions. The low invasiveness of the Mexican strains of virus used in the vaccination experiments described with the resultant incomplete reaction of the control groups of cattle made the interpretation of the results difficult. Nevertheless 88 of a total of 96 cattle *i.e.* 92% which had received a dose of 100 cc of blood vaccine (with crystal violet added), in which the method of inactivation of the virus is by incubation at 37°C, showed no lesions in contact tests or no secondary lesions in tongue inoculation tests when the challenge strain or strains of virus (bivalent vaccines) were those used for preparing the vaccine. Even if all tests are included *i.e.* tests in which the doses of vaccine were 100 cc or only 30 cc or 25 cc or the challenge Mexican strain of virus was not the same as that used for vaccine production 110 of 136 *i.e.* 81% showed equivalent protection. This compared with the 12 of 56 *i.e.* 21% of non-vaccinated control cattle which remained free of all lesions in contact tests and secondary lesions in tongue inoculation tests.

As a comparison a series of observations (unpublished) with strain 119 made in a similar way may be cited. In these experiments 72 of a total of 72 cattle *i.e.* 100% injected with 100 cc of a strain 119 vaccine remained free of all lesions in contact tests and secondary lesions in tongue inoculation tests when tested with strain 119. If 68 cattle which received only 30 cc of vaccine are added, 133 of a total of 140 *i.e.* 95% similarly remained free of lesions. These figures can be compared with 2 of a total of 88 *i.e.* 2.3% of animals in the unvaccinated control groups which similarly remained free of lesions.

These figures indicate the effectiveness of the type of vaccine employed in these experiments and give some idea of the good immunogenicity of the Mexican strains of virus.

The difference in antigenic behavior between the Mexican strains M.1 and MP was not as clear cut as those recorded in the complement fixation and serum neutralization tests.

More comparative data are required to interpret the significance of the antigenic dissimilarity between strains indicated by the different methods of examination.

It is noteworthy that although the Mexican strains under review were clearly classifiable in the Vallée A immunological group of foot-and-mouth disease virus, a potent vaccine prepared from the Pirbright stock Vallée A type strain 119 did not protect cattle against infection with strain MP virus under the conditions of the experiment.*

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* Since this paper was submitted for publication the question of whether a large dose of a strain 119 vaccine would mask this antigenic dissimilarity has been investigated further. The opportunity was taken also to study the antigenic relationship of strain 119 and strain M.1. In a tongue inoculation experiment three groups of eight cattle vaccinated respectively with 100 cc doses of a strain 119 vaccine were inoculated intradermally in the tongue 2½ weeks later with strain 119, strain MP and strain M.1 viruses respectively. In the group tested with strain 119 (10,000 I.D. 50) six animals showed no reaction and two had primary lesions with no development of secondary lesions. In the group tested with strain MP (2,500 I.D. 50) and strain M.1 (2,500 I.D. 50) all of each group of eight animals developed primary lesions and, in each case, five of the eight developed secondary lesions; 1 foot, 2, 2, 4 and 4 feet respectively in the MP group and 2, 4, 4, 4 and 4 feet in the M.1 group. All the cattle in the unvaccinated control groups showed complete devel-

This result emphasizes the necessity in attempting prophylactic control of Mexican virus infection, of testing vaccines prepared from Vallée A strains other than selected Mexican strains under controlled experimental conditions before using them in the field.

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opment of primary and secondary lesions; strain 119, 16 cattle; strain MP, 8 cattle; strain M.1, 8 cattle.

This result shows that a large dose of a strain 119 vaccine is insufficient to mask the antigenic dissimilarity between strain 119 and the Mexican strains MP and M.1 and that the tongue inoculation test as well as the contact exposure test can be used to demonstrate these differences in antigenic behavior.

16623

Effect of BAL on Cobalt-induced Polycythemia in Rats.

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Introduction. In preparation for a study of the effect of BAL (British Anti-Lewisite) on the biological effects and the excretion of radioarsenic (As^{76}), an experiment was designed to determine the influence of this chemical agent on the development of cobalt-induced polycythemia in rats. The mechanism of cobalt-induced polycythemia is not understood.

Barron *et al.*[1] demonstrated a decreased respiration (Warburg) of hemopoietic tissue from cobalt-stimulated animals. This was not corroborated by Warren *et al.*[2] Recently Burk *et al.*[3-5] have shown that the toxic action of cobalt is related to its effect on –SH groups. It seemed worthwhile, therefore, to determine whether or not BAL, which is effective in the protection of –SH groups from arsenical poisoning, would prevent cobalt-induced polycythemia in rats.

Material and Methods. Young Sprague-Dawley rats weighing approximately 300 g each were divided into 5 groups and prepared as follows: Group I (5 rats) served as a control; Group II (5 rats) received 0.2 mM/kg of BAL in peanut oil intramuscularly 3 times weekly; Group III (15 rats) received cobaltous chloride subcutaneously 5 times weekly (0.357 mg calculated as Co); Group IV (5 rats) received 0.2 mM/kg of BAL 3 times weekly; and Group V (5 rats) received cobalt as above but the BAL was begun 6 weeks after the cobalt was initiated.

Two control hemoglobin and hematocrit (Kato) determinations were made on all rats prior to starting the BAL or cobalt injections and at biweekly or monthly intervals thereafter.

Results. As is indicated in Fig. 1 and 2 the animals which were given BAL and cobalt from the beginning of the experiment, the animals which were given cobalt from the beginning and BAL 6 weeks later, and the animals which were given only cobalt developed a comparable polycythemia (increase in hemoglobin and hematocrit values) in approximately 8 weeks. The hemoglobin and hematocrit values of control animals and animals that received BAL only remained essentially constant.

Discussion. The dose of BAL used in this

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[1] Barron, A. G., and Barron, E. S. G., Proc. Soc. Exp. Biol. and Med., 1937, **35**, 407.

[2] Warren, C. O., Schubmehl, G. S., and Wood, I. R., *Am. J. Physiol.*, 1944, **142**, 173.

[3] Burk, D., Schade, A. L., Hesselbach, M. L., and Fischer, C., *Fed. Proc.*, 1946, **5**, 126.

[4] Burk, D., Hearon, J., Caroline, L., and Schade, A. L., *J. Biol. Chem.*, 1946, **165**, 723.

[5] Burk, D., Hesselbach, M. L., Fischer, C., Hearon, J., and Schade, A., *Cancer Research*, 1946, **6**, 497.

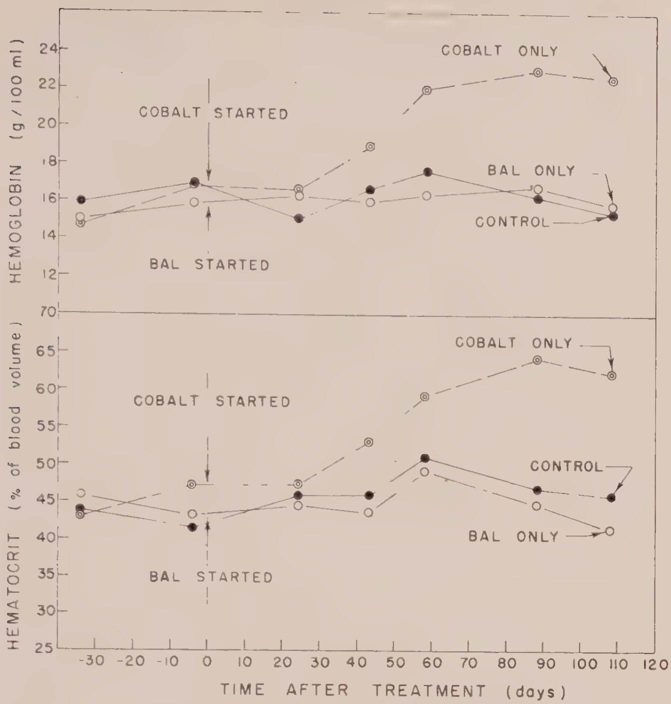

FIG. 1. Effect of 0.2 mM/kg BAL injected intramuscularly 3 times weekly and the effect of cobaltous chloride (0.357 mg calculated as Co) injected subcutaneously 5 times weekly on the hematocrit and hemoglobin values of rats.

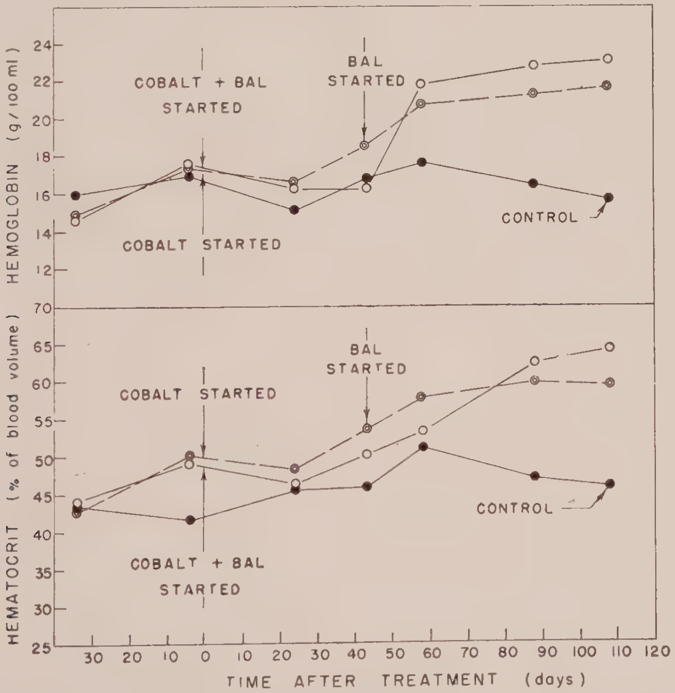

FIG. 2. Effect of 0.2 mM/kg BAL injected 3 times weekly + 0.357 mg cobaltous chloride injected 5 times weekly and the effect of cobaltous chloride + BAL, beginning 6 weeks after cobalt treatment had been initiated, on the hemoglobin and hematocrit values of rats.

experiment has been shown by Olcott *et al.*[6] to be essentially nontoxic for rats when given over a period of 18 days. This dose is 10 times the minimal effective dose for the treatment of acute arsenical poisoning in cats. Since the mechanism of cobalt-induced polycythemia is not known, little can be said concerning the apparent ineffectiveness of BAL to prevent it in rats under the conditions of this experiment. The toxicity of BAL seems to be relatively minimal since administration over a period of 3½ months produced little effect other than slightly retarding weight gain. This experiment indicates that a comparative study of the effect of radioarsenic (As^{76}) on cobalt-induced polycythemia in rats with or without BAL injection is feasible.

Conclusions. BAL in a dose of 0.2 mM/kg given 3 times weekly fails to prevent, cure, or materially alter cobalt-induced polycythemia in rats. The general toxicity of BAL appears minimal when given in this dosage over a 3½-month period.

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[6] Olcott, C. T., and Riker, W. F., *Science*, 1947, **105**, 67.

16624 P

A Fibrillar Structure in Rat Fibroblasts as Seen by Electron Microscopy.

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During the course of comparative tissue culture studies of a strain of normal rat fibroblasts and its malignant cell derivative,[1] we obtained preparations of both of these cell types which under the electron microscope† showed an unmistakable fibrillar structure in the thinly spread cytoplasm. The fibrils, of an estimated thickness of 10 to 100 mμ, may converge and diverge in fan-like formations of great regularity (Fig. 1) but are more often seen gathered together in long bands of varying widths (Fig. 2). The composite bands are seen clearly in phase microscope movies of the cells,[3] but it is only by the increased resolution available with the electron microscope that the nature of the bands becomes clear. They are apparently composed of long thin converging fibrils which in general radiate from the dense central area. That the fibrils are not produced by a wrinkling of the formvar membrane on which the cell is stretched is indicated by their uniformity, by their sharp change in direction in certain areas of the periphery, and by their fine converging and diverging structure. Besides this, no wrinkles were seen in the membrane outside of the cellular area where clear visualization is possible.

Of interest are the relations of these bands to the general structure. The mitochondria, often long and thin, seem frequently to be associated with individual fat droplets. Some of the elongated mitochondria are found in areas which show many microsomes, with the

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* Supported in part by a grant-in-aid from the U. S. Public Health Service.

† Our technic of study of tissue cultured cells is essentially that of Porter[2] with the exception that fixation and staining has been limited to 10 minutes exposure to osmic acid vapors.

We are indebted to Warren A. Hovis for constant careful technical assistance in the preparation of the slides and photographs.

[1] Gey, G. O., *Cancer Research*, 1941, **1**, 737 (abs.); Firor, W. M., and Gey, G. O., *Ann. Surg.*, 1945, **121**, 700.

[2] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

[3] Gey, G. O., Gey, M. K., Firor, W. M., and Self, W. O., *Acta Union Internationale Contre le Cancer*, 1948, in press.

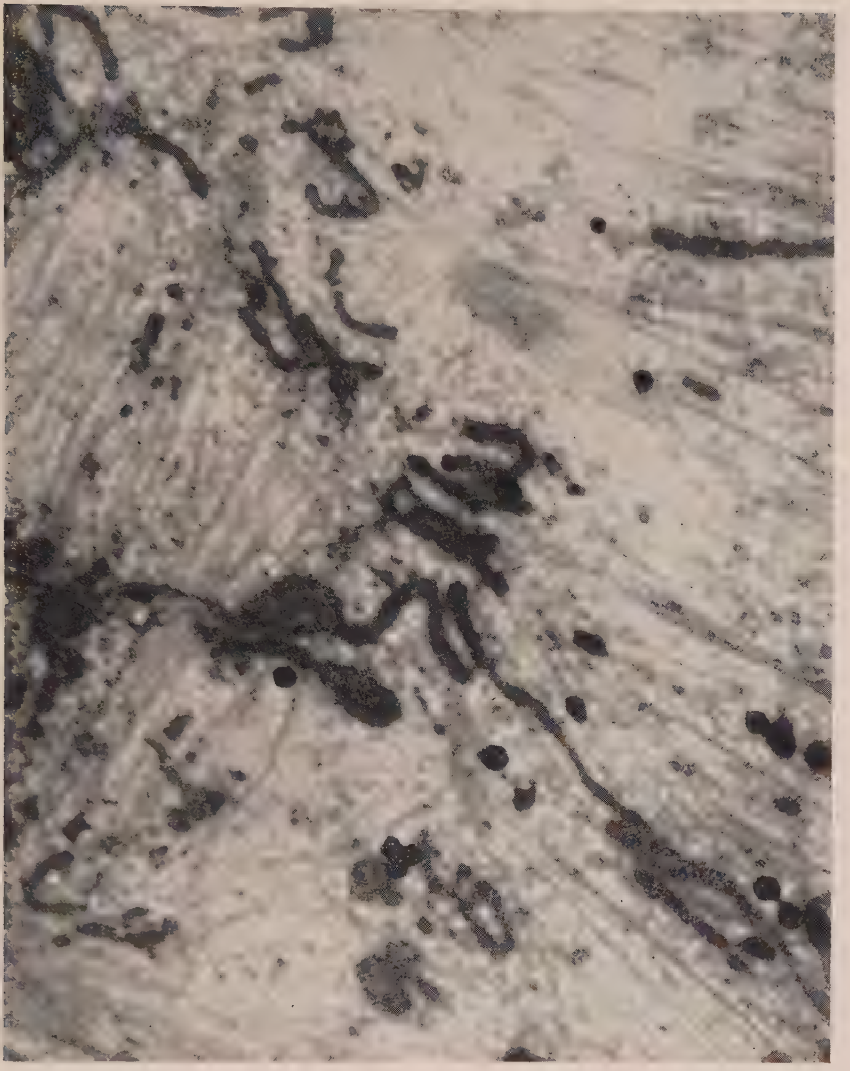

FIG. 1 × 6525.
Large masses of sausage shaped mitochondria are scattered over entire field. Smaller black round bodies are fat droplets. Fibrous structure may be seen criss-crossing throughout picture.

whole area bounded by parallel fibrous bands. This appears to be a static representation of the streams of material which can be seen in the movies of these cells. In other areas, the great masses of mitochondria may be seen crossing those fibrous bands.

This fibrillar structure when present is clear and definite but is not present in every cell and thus may well represent a phase in either the growth or migration of the cells. Such structures have not previously been described in electron microscope studies of similar cells[4,5‡] and many types of cells will have to be studied before their general significance can be established.

Although the above findings cannot in

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[4] Porter, K. R., and Thomson, H. P., *Cancer Research*, 1947, **7**, 431.

[5] Claude, A., Porter, K. R., and Pickels, E. G., *Cancer Research*, 1947, **7**, 421.

[‡] Porter has since stated that the "cytoplasm may show a number of fine fibrils which seem to be differentiations of an otherwise homogeneous cytoplasm," *Anat. Rec.*, 1948, **100**, 72.

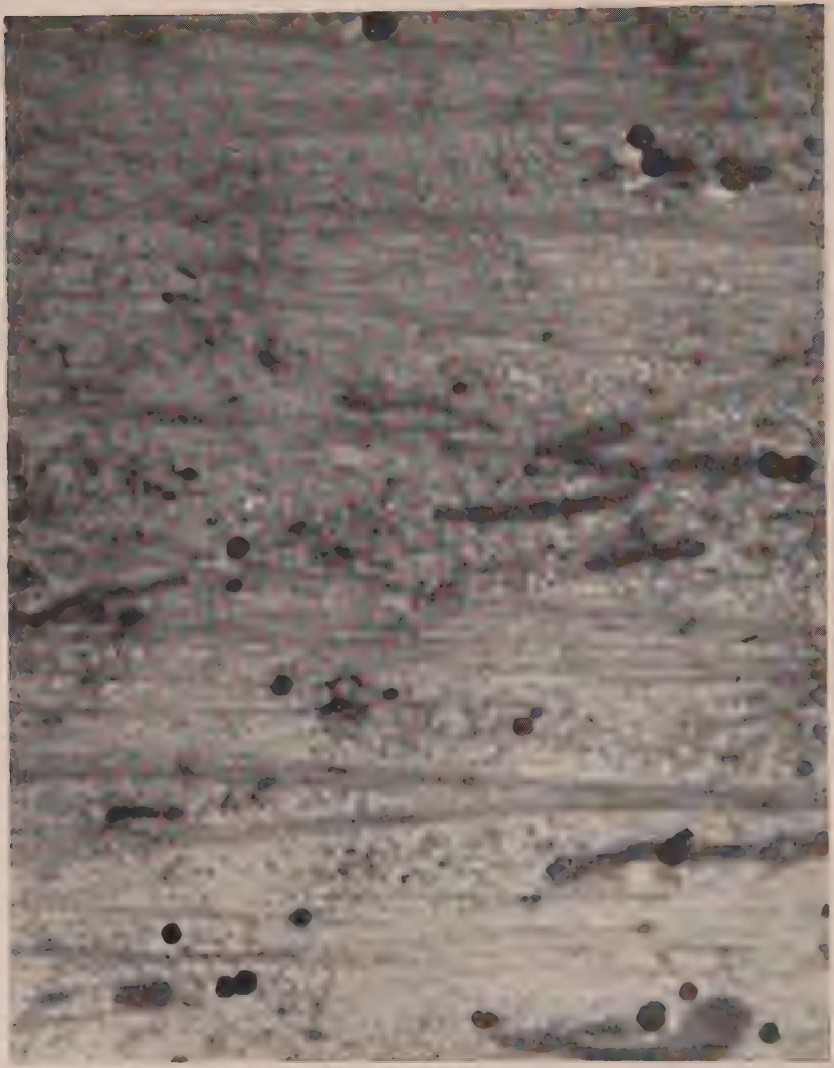

FIG. 2.
Parallel fibrous bands enclosing mitochondria and fat droplets in bed of ground substance.

themselves establish the presence of these fibrils in living cells, electron microscope studies have previously demonstrated fibrillar structure in specialized components of the cytoplasm such as the axons of nerve cells[6] and the tails of sperm cells.[7]

Furthermore, it has been supposed for some time that cytoplasm may have a basic fibrillar structure.[8] This is based on the demonstration of the elasticity of protoplasm (both to externally applied tensions and to internally displaced foreign bodies) and on the viscosity of the cytoplasm. Monné believes that the studies using the polarization microscope "prove that the ground cytoplasm of normal living cells has a fibrillar structure".[9]

The motility of *Amoeba proteus* has been formulated in terms of a continuously changing sol-gel system (Mast)[10] and this concept has been further developed and applied by Lewis[11] to rat fibroblasts. Since gels probably acquire their rigidity from a structure of interwoven fibers, this does not basically differ from the theory of the fibrillar structure of cytoplasm.

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[6] DeRobertis, E., and Schmitt, F. O., *J. Cell. and Comp. Phys.*, 1948, **38**, 1.

[7] Schmitt, F. O., In Advances in Protein Chemistry I., New York, Academic Press, 1944.

[8] Frey-Wyssing, A., Submicroscopic Morphology of Protoplasm and Its Derivatives, New York, Elsevier Publishing Co., 1948.

[9] Monné, L., In Advances in Enzymology VIII., New York, Interscience Publishers, 1948.

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[10] Mast, S. O., *Protoplasm*, 1931, **14**, 321.

[11] Lewis, W. H., In the Structure of Protoplasm. A Monograph of the Am. Soc. of Plan Phys., Iowa State College Press, 1942.

16625 P

The Structure of Unstained Reticulocytes.

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Brilliant cresyl blue and certain other basic dyes, if applied to fluid blood, bring out a reticulum in young red blood cells. This reticulum fails to appear if brilliant cresyl blue or other basic dyes are applied to dried and alcohol fixed blood smears. Moreover, no reticular structure could hitherto be demonstrated in the young red blood cells by microscopic examination of unstained blood with direct or oblique illumination, dark field illumination or in ultraviolet light.[1]

While examining unstained wet blood films of albino mice with the phase microscope, we found that an occasional red blood cell showed a very delicate, hardly discernible reticulum. The cells showing this reticulum were fewer in number than the reticulocytes demonstrable by supravital staining with brilliant cresyl blue. However, when certain hypotonic salt solutions, preferably potassium oxalate 0.8% or ammonium oxalate 1.2% were mixed with the fresh blood, a larger number of cells, corresponding to the percentage of reticulocytes, showed distinct granules and short rods within their cytoplasm.

Most of these rods and granules showed prominent Brownian movement. In such preparation, varying number of cells showed marked loss of hemoglobin. Granules predominated in hemolyzed cells, while rods were more prominent in cells containing ample hemoglobin. The percentage of cells showing rods or granules was higher in those areas of the preparation in which hemolysis was marked. Smears, dried and fixed in alcohol, failed to show these cytoplasmic structures. However, dried but unfixed smears, if mounted in 10% formalin or 1.2% ammonium oxalate, (Fig. 1), showed rods and granules resembling those seen in the fresh preparations. Also, fixation of dried smears in 3% aqueous potassium dichromate did not interfere with the demonstration of the rods and granules. It is noteworthy that both formalin and dichromate applied to the dried smear hemolyzed the red blood cells. Formalin added to fresh blood

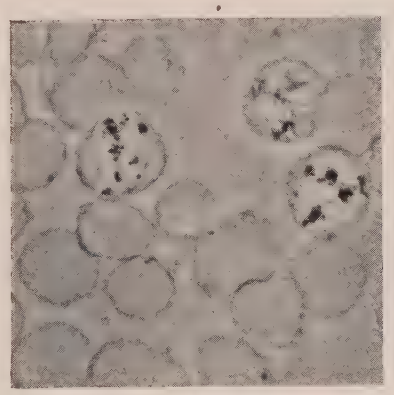

FIG. 1.
Phase illumination of dried film of mouse blood mounted in 1.2% ammonium oxalate.

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[1] Isaacs, R., in Downey, Handbook of Hematology (New York: Paul Hoeber), 1938, **1**, 16.

prevented hemolysis, but also prevented the demonstration of rods and granules when blood so treated was examined in either wet or dried films.

The identity of the cells containing rods and granules with reticulocytes could be demonstrated by adding a small amount of brilliant cresyl blue or new methylene blue[2] to the oxalate solution used in preparing wet films. When this was done, the rods and granules were incorporated into stainable reticulum. Even small amounts of these basic dyes stained the reticulocytes very rapidly and only an occasional cell could actually be observed in the process of staining. From such observations it appeared that some of the granules seen in unstained preparation corresponded to the metachromatic granules seen in the midst of the reticulum when stained with basic dyes.[3] The reticulum, however, appeared frequently independently of the preexisting granules, although these granules may be incorporated into the meshes of the reticulum.

Preliminary experiments demonstrated essentially similar rods and granules in human reticulocytes.

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[2] Brecher, G., unpublished data.

[3] Cesaris-Demel, A., *Folia hemat.*, 1907, **4**, Suppl. 1, p. 1.

16626

Influence of Interrupted Carcinogenic Treatment on Tumor Formation.*

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There is considerable evidence that the formation of cancer occurs as a series of biological changes.[1-5] This concept is supported by several types of experimental data but of special consequence is the evidence of a latent period under certain conditions of carcinogenesis. When a carcinogen is applied to the skin of mice for a time short of that necessary to induce tumors, the subsequent resumption of the carcinogenic stimulus will quickly evoke the formation of tumors even though 2 to 3 months intervene between the 2 periods of treatment.[6] Actually a noncarcinogenic agent, such as heat, a wound or croton oil, may substitute for the carcinogen in the second period.[7-8] Such observations indicate that the initial application of the carcinogen produces alterations in the cells that persist for a considerable period. Whether the initial cellular changes revert gradually or persist indefinitely is not known. In order to test this point, rest periods from one to 3 months were interspersed between 2 periods during which the carcinogens were applied to mice at minimal carcinogenic levels. It was thus possible to obtain some information on the reversibility of the process.

Procedure. This investigation may be divided into two parts: in one, methylcholanthrene was employed to initiate the process

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* This investigation was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council and by a grant from the Jonathan Bowman Fund for Cancer Research.

[1] Berenblum, I., and Shubik, P., *Brit. J. Cancer*, 1947, **1**, 383.

[2] Friedewald, W. F., and Rous, P., *J. Exp. Med.*, 1944, **80**, 101.

[3] Rusch, H. P., *Physiol. Rev.*, 1944, **24**, 177.

[4] Kline, B. E., and Rusch, H. P., *Cancer Res.*, 1944, **4**, 762.

[5] Rusch, H. P., and Kline, B. E., *Arch. Path.*, 1946, **42**, 445.

[6] Lavik, P. S., Moore, P. R., Rusch, H. P., and Baumann, C. A., *Cancer Res.*, 1942, **2**, 189.

[7] Des Ligneris, M. J. A., *Am. J. Cancer*, 1940, **40**, 1.

[8] Berenblum, I., *Arch. Path.*, 1944, **38**, 233.

and, after varying periods of rest, croton oil, a non-carcinogenic agent, was used to evoke the neoplasms; in the other ultraviolet irradiation was used for both periods of treatment.

Methylcholanthrene - croton oil. Young adult strain C mice of both sexes were used. They were kept on shavings in groups of 20-24 in metal box cages with a stock grain ration and water available at all times. A solution of 0.2% methylcholanthrene in benzyl alcohol was painted on the backs of all mice twice weekly for 19 weeks. At this time approximately 10% of the animals had papillomas. The mice with papillomas were discarded and those free of visible tumors were divided into 6 groups of 42. These were then treated as follows: One group received no further treatment, another had the hydrocarbon applications continued for 13 weeks, and in a third group a 0.5% solution of croton oil in benzyl alcohol was applied to the precarcinogenic area, 3 times a week for thirteen weeks. The remaining 3 groups were also painted with croton oil in the same manner as the latter group except that rest periods of 4, 9, and 13 weeks elapsed between the cessation of methylcholanthrene application and the start of the treatment with croton oil. The incidence of tumors was checked every 2 weeks and they were classified as papillomas and carcinomas.

Ultraviolet Irradiation. Young adult albino strain C mice of both sexes were used. They were kept on shavings in groups of 24 in ordinary metal box cages with a stock grain ration and water available at all times except during irradiation. At the time for raying, they were transferred to a special cage 25.5 cm square by 3 cm deep, constructed of wire mesh, and divided into 24 individual compartments to prevent the mice huddling together and to minimize movements.[9] Except for rest periods, all mice were irradiated 30 minutes a day, 6 days a week with the light of a medium pressure mercury vapor lamp. The daily amount of irradiation was 3.6 x 10^7 ergs/cm^2 at an intensity of 2 x 10^4 ergs/cm^2

TABLE I. Effect of Rest Periods on Incidence of Carcinomas Following Treatment with Methylcholanthrene and Croton Oil.
(A solution of 0.2% methylcholanthrene (Me) was applied to backs of all these mice for a period of 19 weeks prior to treatment indicated below).

Group	Subsequent	Tumor incidence—months after start of first application of methylcholanthrene														
		6			7			8			9			10		
		N	T	%	N	T	%	N	T	%	N	T	%	N	T	%
1	Me continued 3 mo.	41	1	2	38	4	10	25	17	40	12	30	71	1	41	98
2	Croton oil 3 mo.	40	2	5	34	7	17	21	18	43	11	28	67	2	35	85
3	Rest 1 mo. then Croton oil 3 mo.	42	0	0	40	2	5	32	8	19	16	21	50	8	28	67
4	Rest 2 mo. then Croton oil 3 mo.	42	0	0	40	2	5	35	7	17	21	19	45	12	27	65
5	Rest 3 mo. then Croton oil 3 mo.	42	0	0	38	4	10	30	8	20	23	14	33	18	16	38*
6	No treatment	42	0	0	41	1	2	34	5	12	27	9	21	21	14	33

N = Free of malignant neoplasms. T = Malignant neoplasm.

* At 11 months there were: N 15, T 18, % 43

The per cent of tumors is calculated from the number of animals alive at the time the first tumor is noted.

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[9] Rusch, H. P., Kline, B. E., and Baumann, C. A., *Arch. Path.*, 1941, **31**, 135

/sec. The mice were examined for tumors every 2 weeks and were weighed at monthly intervals. Details of procedure for each group are given in Table II.

Results. Methylcholanthrene-Croton Oil. The rates of tumor formation in the various groups of mice are listed in Table I. At the end of 19 weeks of treatment with methylcholanthrene about 10% of the mice had developed papillomas. At this time, the applications of hydrocarbon were stopped and all the animals with visible tumors were discarded. The remaining mice were divided into 6 groups of 42 each and were treated according to the method indicated in Table I.

The formation of papillomas continued at a rapid rate in all groups, and one month after the initial methylcholanthrene had been discontinued, less than half of the mice in all groups were free of benign tumors. The rate of formation of the malignant neoplasms was slower and varied considerably among the groups. In the mice that received no further treatment, the rate was steadily upward and 33% of the animals had carcinomas at the end of 10 months (Group 6). The rate of development of malignant tumors was very rapid in the mice of the control group in which the hydrocarbon was continued for another 3 months (Group 1) and in the mice that were painted with croton oil immediately after the initial period (Group 2). The final incidence of tumors after 10 months in these latter 2 groups was 98 and 85% respectively. In confirmation of the earlier observation of Berenblum[8] croton oil was almost as effective as methylcholanthrene in eliciting tumors in a precarcinogenic area. When periods of one or 2 months were allowed to elapse before the application of croton oil, the development of carcinomas was delayed slightly (Groups 3 and 4), but the rate of tumor formation paralleled that in Groups 1 and 2 following the treatment with the irritant. The final tumor incidence in groups 3 and 4 at 10 months was 67 and 65% but the slope of the curve indicated an eventual incidence close to that in Groups 1 and 2. When three months elapsed between the initial treatment and the start of croton oil, the rate of formation of

TABLE II.
Effect of Rest Periods on Tumor Incidence Following Treatment with Ultraviolet Irradiation.

Group	Procedure (Time in months)	Tumor incidence—months after start of raying																				
		4			5			6			7			8			9			10		
		N	T	%	N	T	%	N	T	%	N	T	%	N	T	%	N	T	%	N	T	%
A	Ray 5	22	2	8	18	5	21	10	12	50	4	18	75	0	21	88	—	—	—	—	—	—
B	,, 3, Rest 1, Ray 2	23	0	0	22	1	4	13	9	39	3	18	78	2	19	83	—	—	—	—	—	—
C	,, 3, ,, 2, ,, 2	21	1	5	17	2	9	14	5	23	6	10	45	4	11	50	3	12	55	—	—	—
D	,, 3, ,, 3, ,, 2	24	0	0	23	1	4	20	1	4	17	3	13	12	7	29	6	12	50	5	13	54
E	,, 3, ,, 1, ,, 1 ,, 1, ,, 1	24	0	0	19	4	17	17	6	26	11	12	52	2	19	83	—	—	—	—	—	—
F	,, 3, ,, 1, ,, 1	23	1	4	19	4	17	13	10	42	8	15	63	4	19	79	—	—	—	—	—	—
G	,, 1, ,, 1, ,, 2	23	1	4	23	1	4	19	1	4	19	1	4	18	2	8	11	5	21	—	—	—
H	,, 1, ,, 1, ,, 1	24	0	0	23	0	0	23	0	0	23	0	0	22	0	0	19	0	0	—	—	—
I	,, 1	24	0	0	24	0	0	23	0	0	22	0	0	22	0	0	20	0	0	—	—	—
J	,, 2	23	0	0	21	1	5	20	1	5	20	1	5	18	2	9	15	2	9	—	—	—
K	,, 3	24	0	0	23	1	4	20	2	8	20	2	8	18	3	13	12	5	21	—	—	—
L	,, 4	24	0	0	17	6	26	13	10	44	8	13	57	5	15	65	—	—	—	—	—	—

Designations are the same as in Table I.

neoplasms was slower and the eventual number of tumors obtained was less than in the other groups; the incidence at the end of 11 months being 43%.

Ultraviolet Irradiation. The rate at which tumors appeared in the various groups of mice irradiated with ultraviolet light is given in detail in Table II. The animals remained in good health throughout the experiment and the tumors formed were identical to those previously reported.[10] Mice rayed continuously for 5 months developed tumors rapidly and there was an incidence of 88% after 8 months (Group A). In the next 3 groups the 5 months of irradiation was divided into two periods with an initial irradiation of 3 months separated from the last 2 months by rest periods of one, 2 or 3 months. A rest of one month had little effect on tumor formation, and the results were essentially the same as for the control group (Group B). However, when the rest period was prolonged to 2 or 3 months, the incidence of neoplasms at 9 months was only 55 and 50% respectively (Groups C and D), but the rate of tumor formation in these groups was similar to that in Groups A and B. The rapid response to the second period of raying is evident from the data.

The effect of various other periods of irradiation or rest were also observed. When the total 5 months' irradiation was divided so that the initial 3 months of raying was followed by one of rest, one of ray, another of rest and a final one of irradiation (Group E), the results were similar to those obtained when the initial period was followed by one of rest and 2 of irradiation (Group B). Essentially no difference was seen when the final period of irradiation was only of one month duration instead of 2 (Group F). When 3 months of irradiation were divided so that the initial period of one month was separated from the final period by a month of rest, (Group G), the results were similar to those obtained when they received the same total dose over a continuous period (Group K).

Groups H, I and J illustrate that a minimal amount of irradiation was required to induce tumors. Since this fact had been previously known these groups were employed only for controls of the present experiment. One month of irradiation by itself was insufficient to elicit tumors during the period of observation (Group I). The practical minimal time under the condition of this experiment was 2 months of continuous irradiation (Group J) and the same total dosage divided into 2 equal periods but separated by a month of rest was ineffective (Group H). Mice rayed for 3 months had a tumor incidence of 21% at the end of 9 months (Group K), and 4 months of continuous irradiation (Group L) was almost as effective as 5 months. In retrospect, it appears that the lower incidence of tumors obtained with only 2 months of irradiation would have been a more satisfactory initial dose for testing the effect of rest periods than the longer period used in this experiment. The longer initial period was selected on the basis of previous work that indicated a two month period to be a marginal one.

Discussion. The present investigation strengthens our previous suggestions that the process of tumor formation can be divided into 3 periods.[4,5] The initial phase is a conversion of a few normal cells into a few neoplastic cells and has been called the Period of Induction[4,5] or the Initiating Process.[1,2] The next stage is the multiplication of such cells into growing visible tumors, and it is the division of this period into two separate stages that we wish to emphasize. The first of these periods is the Critical Period.[5] This starts when the genesis of the neoplastic cell has been completed and is that stage in which cellular proliferation is delicately balanced. During this phase there are so few neoplastic cells present that they are more or less lost among the normal cells, and since their mass has not yet attained a size sufficient to receive a direct supply of blood, they must compete with the healthy cells for the nutrients in the tissue spaces. At such times these cells are the most susceptible to the influence of their environment; they may proliferate, lie dormant, or succumb, or all three processes may occur in the same general area at one time. The fate of small masses of neoplastic tissue depends on the balance between their proliferative capacity and the amount of stimulation or of resistance present in the neighboring tissues. The sum of all these factors determines the rate at which the cells proliferate.[11,12] If the proliferation of the neoplastic cells continues until they attain a size sufficient to establish an independent blood supply, they then become less dependent on local environmental conditions and enter the third phase—that of Progression.[5]

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[10] Grady, H. G., Blum, H. F., and Kirby-Smith, J. S., *J. Nat. Cancer Inst.*, 1943, **3**, 371.

The postulation of a critical period appears essential for the explanation of the results presented in this paper. In the first place, a period of reversibility is indicated because of the decreased incidence of tumors following a long interval between the first and final carcinogenic treatments. Such results may be explained on the basis of the number of cells surviving during the quiescent period. If the rest period is prolonged, some of the neoplastic cells die so that the number able to respond to the second treatment with irritant or carcinogen diminishes as the period of rest increases. Actually, if the rest period is very long, the latent tumor cells may disappear entirely or be reduced to such a level that no visible tumors could develop in the normal life of the mouse even though the proper stimulus is eventually applied. Furthermore, the evidence indicates that nonspecific irritants are effective only during an interval that corresponds to the critical period described in the preceding paragraph. The irritant cannot convert normal cells into malignant ones[1] and since it also is without effect on the growth of visible tumors, it can be assumed that the action is exerted during an intermediate period. The conversion of normal cells into malignant ones is brought about solely by the specific carcinogenic agents,[1] an inference that is borne out by the rapidity by which tumors are evoked following the treatment by croton oil. Evidence from other sources also favors this concept.[2,5,11,12]

Indications for a critical period in this investigation are in contrast to those of Berenblum and Shubik[1] who find that the initial changes are irreversible. They used only one application of 9:10 dimethyl-1:2-benzanthracene and reported only the appearance of warts, whereas we used multiple treatments with methylcholanthrene or ultraviolet irradiation and include only the malignant neoplasms in the data of this paper. Since benign tumors proliferate more slowly it is possible that their life span is longer. To test this point the experiment of Berenblum and Shubik should be repeated allowing a greater period of rest before the irritant is applied.

Summary. The influence of interrupted carcinogenic treatment on tumor formation was tested. Rest periods of from one to 3 months were interspersed between 2 periods during which the carcinogens were applied to mice at minimal carcinogenic levels. The investigation was divided into two parts on the basis of the carcinogens employed. In one, ultraviolet irradiation was used for both the first and second periods. In the other, methylcholanthrene was employed to initiate the process and croton oil, a non-carcinogenic substance, was used to evoke the neoplasms. The results with both technics were essentially the same.

A lag in tumor appearance was observed during the rest periods, and the second period of treatment quickly brought forth tumors in the precarcinogenic areas. Essentially no difference in the rate of tumor formation or in the final tumor incidence was observed when the rest period was of one month duration. When the rest period was increased to 3 months, the final tumor incidence was reduced by 34% in the mice irradiated and by 42% in those in the methylcholanthrene-croton oil series. The results indicate that the initial application of the carcinogen produced alterations in the tissues which were maintained for at least one month, thereafter the changes re-

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[11] Blum, H. F., *J. Nat. Cancer Inst.*, 1943, **3**, 569.

[12] Blum, H. F., *J. Nat. Cancer Inst.*, 1944, **4**, 559.

verted toward normal. It is suggested that the genesis of the neoplastic cell was completed during the initial treatment, and such cells remained dormant or divided at a decreased rate until further stimulated. The non-specific irritant, croton oil, exerts its effect during the critical period of carcinogenesis.

16627

Antigenic Relationship of *Salmonellae* to Inaba Strains of *Vibrio comma* Isolated in Egypt.

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The sera of patients infected with *Salmonellae* frequently agglutinate cholera vibrios.[1] Gohar and Makkawi,[2] studying *Vibrio comma* strains from the recent outbreak in Egypt, found that these organisms were agglutinated by *Salmonella enteritidis* sera. It seemed of interest, therefore, to investigate which antigenic factors are common to *Salmonellae* and cholera vibrios.

Three strains of *V. comma* of the Inaba type, isolated from the recent cholera epidemic in Egypt, were used in these experiments.* *Salmonella* "O", "Vi" and "H" sera prepared for routine *Salmonella* grouping (typing) by methods described by Edwards a.o.[3-5] were employed. Procedures recommended by Ranta and Dolman[6] were used for the production of *V. comma* "OH" sera. Agglutination and absorption tests were carried out according to the standard methods employed for *Salmonella* grouping (typing).[4,5]

Live, alcohol-treated ("O") and formolized ("H") antigens prepared from the cholera vibrios were tested against 25 "O", 2 "Vi" and 39 "H" *Salmonella* sera. Agglutination of the vibrios occurred with dilutions of these

TABLE I.
Results of Agglutination Tests Using *Salmonella bredeney* ''O'' Serum.

Agglutination (after absorption) with ''O'' antigen from	Serum absorbed with ''O'' antigen from							
	None	*S. bredeney* I, IV, XXVII, XII	*S. paratyphi* A I, II, XII	*S. pullorum* IX, XII	*S. worthington* I, III, XXIII	*V. comma*		
						''k''	''4''	''8''
S. bredeney I, IV, XXVII, XII	16*	0	6	4	4	12	12	12
S. paratyphi A I, II, XII	8	0	0	4	2	4	2	2
S. pullorum IX, XII	6	0	0	0	6	1	2	2
S. worthington I, III, XXIII	5	0	1	5	0	3	2	2
V. comma ''k''	2	0	0	1	1	0	0	0
V. comma ''4''	4	0	0	1	1	0	0	0
V. comma ''8''	2	0	0	2	1	0	0	0

* All agglutination titers to be multiplied by 100.

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[1] Felsenfeld, O., and Young, V. M., A.A.A.S. Annual Meeting, 1946.

[2] Gohar, M. A., and Makkari, M., *J. Trop. Med. and Hyg.*, 1948, **51**, 95.

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TABLE II.
Results of Agglutination Tests Using *Salmonella California* "H" Serum.

Agglutination (after absorption) with "H" antigen from	Serum absorbed with "H" antigen from							
						V. comma		
	None	*S. california* g, m, t	*S. oranienburg* m, t	*S. essen* g, m	*S. senftenberg* g, s, t	"k"	"4"	"8"
S. california, g,m,t	120*	0	20	5	20	80	80	80
S. oranienburg, m,t,	60	0	0	5	10	60	60	60
S. essen, g,m	40	0	20	0	5	20	25	20
S. senftenberg, g,s,t	30	0	10	5	0	10	12	10
V. comma "k"	6	0	5	0	0	0	0	0
V. comma "4"	4	0	4	0	0	0	0	0
V. comma "8"	6	0	6	0	0	0	0	0

* All agglutination titers to be multiplied by 100.

TABLE III.
Average of Results of Agglutination Tests Using *Vibrio comma* "OH" Sera.

Agglutination (after absorption) with antigen from	Serum absorbed with antigen from							
	None	*S. bredeney* I, IV, XXVII, XII	*S. pullorum* IX, XII	*S. worthington* I, III, XXIII	*S. california* g,m,t	*S. oranienburg* m,t	*S. essen* g,m	*S. senftenberg* g,s,t
V. comma "k"	14*	8	10	10	8	14	9	9
V. comma "4"	12	8	9	9	8	12	8	8
V. comma "8"	12	8	9	9	7	12	7	8
S. bredeney I, IV, XXVII, XII	2	0	1	1	2	2	2	2
S. pullorum IX, XII	2	0	0	2	2	2	2	2
S. worthington I, III, XXIII	2	0	2	0	2	2	2	2
S. california g,m,t	4	4	4	4	0	4	0	0
S. oranienburg m,t	0	0	0	0	0	0	0	0
S. essen g,m	4	4	4	4	0	4	0	0
S. senftenberg g,s,t	4	4	4	4	0	4	0	0

After absorption with *V. comma* antigens, all agglutination tests were negative.
* All agglutination titers to be multiplied by 100.

sera as high as 5 to 25% of their titer against the homologous *Salmonella* when the serum contained agglutinins for the *Salmonella* antigenic factors I, XII or g. Subsequent absorption tests, as represented in Tables I and II confirmed that parts of *Salmonella* I, XII and g antigens were present in the *V. comma* strains.

Sera prepared against the *V. comma* strains agglutinated *Salmonellae* which contained the antigenic factors I, XII and g. Results of the absorption tests are given in Table III.

Summary. Three Inaba strains of *V. comma* isolated recently in Egypt harbored fractions of *Salmonella* I, XII and g antigens.

16628

Effect of Rutin on Dicoumarolism.*

William G. Clark.

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As far as the writer is aware, no drug has been described which exerts a specific antagonistic action on the hemorrhagic symptoms of dicoumarolism.

Dicoumarol has been shown to increase bleeding time and this is not correlated with increased prothrombin time.[1,2] Capillary fragility, moreover, is not altered in patients with hemorrhagic symptoms after excessive dicoumarol.[2,3] Dicoumarolism also causes marked dilation of capillaries, small arteries and veins.[4,5] Other toxic vascular changes include marked swelling and vacuolization of endothelium, with areas of endothelium proliferation, degeneration of vascular smooth muscle, perivascular serous exudation and diapedesis of blood cell elements and even necrosis and rupture of the entire vessel wall.

Flavonoid derivatives ("vitamin P"-like substances) have been shown to decrease bleeding time,[6,7] capillary permeability and fragility. Rutin is a well-known flavonoid described as having anti-capillary fragility action by Sevin,[8] later by Griffith, Couch and Lindauer[9] and since then by many others. Widespread clinical application of rutin has resulted, with conflicting reports. An extensive literature exists[10] on the purported anti-hemorrhagic properties of flavonoids and related substances in diverse pathologic states, both experimental and clinical. The mechanism of such action, if true, is not clear, but may be related to either reduction in the size of the capillary bed,[11] or to an antihyaluronidase action.[12-15]

Prandoni and Wright[2,3] noted no effect of "vitamin P" in the form of grated orange peels orally administered to patients suffering hemorrhagic symptons from dicoumarol therapy. The quantity of flavonoids administered by this method is small and the nature of the flavonoids contained in orange peel is largely unknown other than hesperidin and eriodictyol.

Naghski, Copley and Couch[16] reported that the flavonol glycosides, rutin and quercitrin, and quercetin (aglucone of rutin) exert an antagonistic effect against the bacteriostatic effect of dicoumarol and therefore suggested the possibility of using rutin or similar flavonols to antagonize the hemorrhagic action of dicoumarol *in vivo*.

The present note presents evidence which indicates that rutin has no protective action against dicoumarolism in rats.

Experimental. Male albino rats (Slonaker

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* Supported by a grant from the United States Public Health Service.

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strain) were divided into 2 groups of 10 each, averaging 137 g body weight and 2 months of age. Both groups were fed Purina Laboratory Chow mash in which was incorporated 0.04% dicoumarol.† One group received this diet alone and the other received the same diet containing 2% rutin‡ in addition to the dicoumarol. From the average daily food intake it was estimated that each rat ingested about 0.3 mg dicoumarol daily, and the rutin-fed animals ingested about 150 mg rutin daily. Later, as the animals became poisoned, less food was consumed. Records were kept of the hemorrhagic symptoms, body weight loss and survival.

The experiment was terminated after 30 days, at which time there were no survivors in the group fed dicoumarol alone, with an average survival time of 15 days (range 7 to 19); and 10% surviving in the group fed rutin + dicoumarol, with an average survival time of 13 days (range 5 to 21), of the rats which died.

In a second experiment of 10 rats per group, average body weight 120 g, the dietary dicoumarol was adjusted to 0.02% instead of 0.04% as in the first experiment, and the rutin, as before, remained at 2%. The experiment was terminated after 45 days, at which time 90% of the controls survived (the one death occurred in 45 days), and 60% of the rutin-fed animals survived (the 4 deaths occurred in 7, 22, 41 and 44 days).

In a third experiment of 10 rats each, average body weight 140 g, the animals were fed Purina Chow and the dicoumarol ± rutin were stomach-tubed daily as aqueous suspensions in doses of 5 mg dicoumarol ± 50 mg rutin. After a week, the dose of dicoumarol was increased to 10 mg, since 5 mg seemed insufficient to evoke hemorrhagic symptoms. The experiment was terminated in 35 days, at which time 30% of the controls were surviving, with an average survival time of 26 days for the rats which died; and 20% of the rutin-fed animals were surviving, with an average survival time of 18 days for the rats which died.

There were no group differences in onset or severity of hemorrhagic symptoms in the 3 experiments.

Conclusions. Under the experimental conditions employed, rutin does not antagonize dicoumarolism in rats. Presumably the defect in the vessel walls or their supporting structures which leads to hemorrhage in dicoumarolism differs from that leading to the various hemorrhagic conditions which rutin and related "vitamin P"-like substances purportedly benefit. These facts, however, need not necessarily preclude the simultaneous use of dicoumarol and "vitamin P" for their separate effects, as illustrated by the report of MacLean and Brabel[17] on the favorable effects of rutin and dicoumarol in various retinal vascular disorders.

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† Kindly supplied by E. R. Squibb & Sons, New York.

‡ Kindly supplied by the S. B. Penick Co., New York.

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16629

Isolation of Mumps Virus from the Blood of a Patient.

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The isolation of mumps virus from the blood of a patient is not known to have been reported. One case is described, the methods of isolation and identification being essentially those employed by others for saliva[1] and for spinal fluid.[2]

Case History. B.M., a boy aged 3 years, 9 months, was admitted to the Children's Hospital of Boston* May 9, 1948, for treatment of cellulitis of the left chest wall incident to a fall. He appeared acutely ill. The temperature was 103°F, an area over the left chest wall was tender and red, and a scarlitiniform rash, thought to be a drug eruption, covered the entire body. Sulfadiazine and penicillin had been administered. Three days later, May 12, the left parotid was firm, swollen and tender. A clinical diagnosis of mumps was made, the temperature at that time being 101°F, pulse 120, and respirations 25. The total leucocyte count on May 11, was 13,800, with 92% granulocytes and 8% lymphocytes; on May 13, it was 10,400, with 52% granulocytes, 38% lymphocytes, and 10% monocytes. Spinal fluid taken May 12 had a trace of globulin, sugar content within normal limits, and no white blood cells. A tuberculin test was positive in 1:1000 dilution of O.T. A marked right parotid swelling appeared on May 16. No signs or symptoms of meningo-encephalitis were noted. Recovery was rapid and the patient returned home May 22.

Isolation of Mumps Virus. Specimens examined for mumps virus were introduced into the amniotic sac of 7-day-old embryonated eggs and amniotic membranes and fluids harvested separately after 6 days incubation at 35°C. Fluid from each egg was examined for virus by macroscopic hemagglutination of hen erythrocytes. Cultures in thioglycollate broth of pooled amniotic fluids from each egg passage showed no bacterial contaminants. The amniotic membranes of egg passages giving no hemagglutination were ground with alundum, pooled with their fluids and passed in 0.2 cc amounts intra-amniotically. Only positive fluids were pooled and passed when hemagglutination appeared in subsequent passages. Positive amniotic fluids had a bluish opalescence, those showing no hemagglutination were usually clear.

Blood was drawn from patient B.M. on May 12, the day parotitis was first observed and 5 cc treated with sodium citrate to give a final concentration of 0.5%. Because whole citrated blood had resulted in a high mortality in previous egg inoculations, the plasma was separated from the red blood cells after natural sedimentation, and the two fractions stored in carbon dioxide ice. Two weeks later 12 eggs were inoculated intraamniotically with 0.1 cc of the plasma, and 5 survived for harvesting. The red blood cell fraction was diluted 1:2 in infusion broth, and the mixture in 0.2 cc amounts inoculated into 12 eggs intra-amniotically. Only 3 embryos survived. Since the amount of harvested material was small, it was added to the amniotic membrane and fluid pool of the plasma passage. In the third egg passage, virus was detected in 4 of 8 amniotic fluids, and in almost all eggs of 2 subsequent passages.

The cerebro-spinal fluid of May 12, inoculated into eggs in 0.2 cc amounts, yielded no virus in 3 passages; a not unexpected result since the patient had no symptoms suggesting meningo-encephalitis and the spinal fluid contained no leucocytes. Isolation of virus from the saliva was not attempted because of the age and lack of cooperation of the patient.

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[2] Henle, G., and McDougall, C. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 209.

* Acknowledgment is made of the kind cooperation and assistance of the hospital staff; also to the Haynes Memorial Hospital for other materials.

Attempts to isolate mumps virus from the blood of 7 other patients in the first 5 days of the disease were unsuccessful, all but one of the specimens being drawn after the first day of illness. Whole defibrinated blood is less lethal to chick embryo than citrated blood.

Serologic Studies and Identification of Virus. The inhibition of hemagglutination and the complement fixation tests used in identification of mumps virus have been well-described by others.[3] Paired serums from a patient, taken in the acute and convalescent phases of mumps and known to demonstrate an increasing titer of inhibiting antibodies, were kindly furnished by Dr. J. F. Enders. Strain B.M. mumps virus, freshly isolated from the blood and used as an antigen with the standard acute serum gave inhibition of hemagglutination in a titer of 1:16; with the convalescent serum in a titer of 1:256, a 16-fold rise, titers being expressed in terms of initial dilution. The clinical diagnosis of mumps for patient B. M. was confirmed by a similar test, using the Enders strain of mumps virus as antigen against paired serums of the patient.† Serum of May 12 had a titer of 1:32 and that of May 22, 1:256, an 8-fold rise in titer.

Identification of the virus as that of mumps was further substantiated by test for fixation of complement with pooled amniotic fluids of the sixth egg passage as antigen. Paired sera from a patient with mumps, and with demonstrated increasing complement fixing antibodies (Diagnostic Laboratory, Massachusetts Department of Public Health) were used as a standard. The acute phase serum had a titer of 1:16 and the convalescent serum 1:256.

A complement fixation test of serums from patient B. M., using the Enders strain of mumps virus as antigen, gave a titer of 1:32 with acute phase serum and a four-fold rise to 1:128 with convalescent serum.

Isolations from Spinal Fluid. Successful isolation of mumps virus has been accomplished in 5 or 7 cases of meningo-encephalitis, all of which demonstrated a rise in complement fixing antibodies during convalescence, where spinal fluids were examined within 5 days of onset. An 8-fold or greater rise of titer in the inhibition of hemagglutination test was used in identification of the viruses isolated.

Discussion. A virus was isolated from the blood of a patient on the first day of a parotid swelling conforming to that of mumps. The clinical diagnosis was confirmed by a significant rise in titer of mumps complement-fixing and inhibition of hemagglutination antibodies in the course of convalescence. Two methods, inhibition of hemagglutination and complement fixation, identified the virus as that of mumps.

That mumps virus invades the blood stream is not surprising, in view of the considerable amount of virus appparently present in an infected parotid gland. The regularity of such invasion and its significance in the pathogenesis of the disease, awaits the result of studies now in progress.

Summary. The virus of mumps was isolated from the blood of a patient with bilateral parotid swelling through culture in embryonated eggs. Inhibition of hemagglutination and complement fixation tests confirmed the clinical diagnosis and identified the agent as the mumps virus.

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[3] Diag. Procedures for Virus and Rickettsial Diseases. Am. Pub. Health Assn., 1948, p. 139.

† Test was kindly performed by the Research Division of Infectious Diseases, Children's Hospital, Boston.

16630

Relative Lack of Myelin in Optic Tracts as Result of Underfeeding in the Young Albino Rat.*

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The underfeeding experiments reported herein were originally started, and are still being carried on, to determine the effects of inanition on the ability to regulate body-temperature. We have previously presented experimental evidence indicating that normal myelination of hypothalamic fibers of young rats may have an important bearing upon the attainment of regulatory ability.[1] The myelin lack in the optic tracts of underfed animals has been an incidental finding, but one which may have considerable practical importance.

Donaldson[2] studied the effects of underfeeding on myelination in the central nervous system of the albino rat. His rats were 30 days old at the beginning of the experiment and 51 days old when sacrificed. The average percentage difference in body weight, at the end of the experiment, between his control and underfed animals was 41.2, and the average percentage difference in brain weight was 7.7. Weigert-stained sections did not show grossly observable differences between the myelin content of the brains from the control and those from underfed rats. Donaldson concluded from these studies that underfeeding does not arrest myelination.

Methods and Materials. Six litters of Wistar strain albino rats have been used in the present investigation. On the 17th to 20th day of life, 3 rats from each litter were weaned, and placed in individual cages, where each was maintained on a diet of 5 g of Purina laboratory chow per day. The control animals were left with their mothers until they had reached 26 days of age, when they were weaned and then maintained on Purina laboratory chow *ad libitum.* The underfed animals and their litter-mate controls were sacrificed on the 50th to the 52nd day of life.

When sacrificed, the experimental and control animals were perfused with Regaud's solution (3½% potassium dichromate—80 parts; neutral formalin—20 parts). Following perfusion, the brains were removed, weighed, and placed in 5% potassium dichromate. They were left in this solution for 10 days at 38-40°C; the solution was renewed on the 2nd, 5th and 8th days. When all the brains from a given litter had been embedded in paraffin, they were sectioned at 20 micra and mounted on slides. Representative slides from all the animals in the litter were placed together in a staining rack and stained en masse according to the method reported by Ulett, Dow and Larsell.[3]

The concentration of myelin in the optic tract was determined on the basis of monochromatic light absorption by stained sections as previously described.[1] After focusing upon the optic tract the slide was moved to an adjacent area which was free of sections and, without altering this focal adjustment, the amount of light entering the microscope was adjusted by means of a rheostat and the iris diaphragm at the inlet of the monochromater to give a reading of 80.0 on the galvanometer of the electrophotometer. The light was adjusted to this same "zero" reading in relation to each of the determinations in a given series, and this compensated for differences in thickness of slides, cover slips, and mounting media. When the light had been so adjusted, the optic tract was moved back into the field and the galvanometer reading record-

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* Supported by a grant from the Office of Naval Research.

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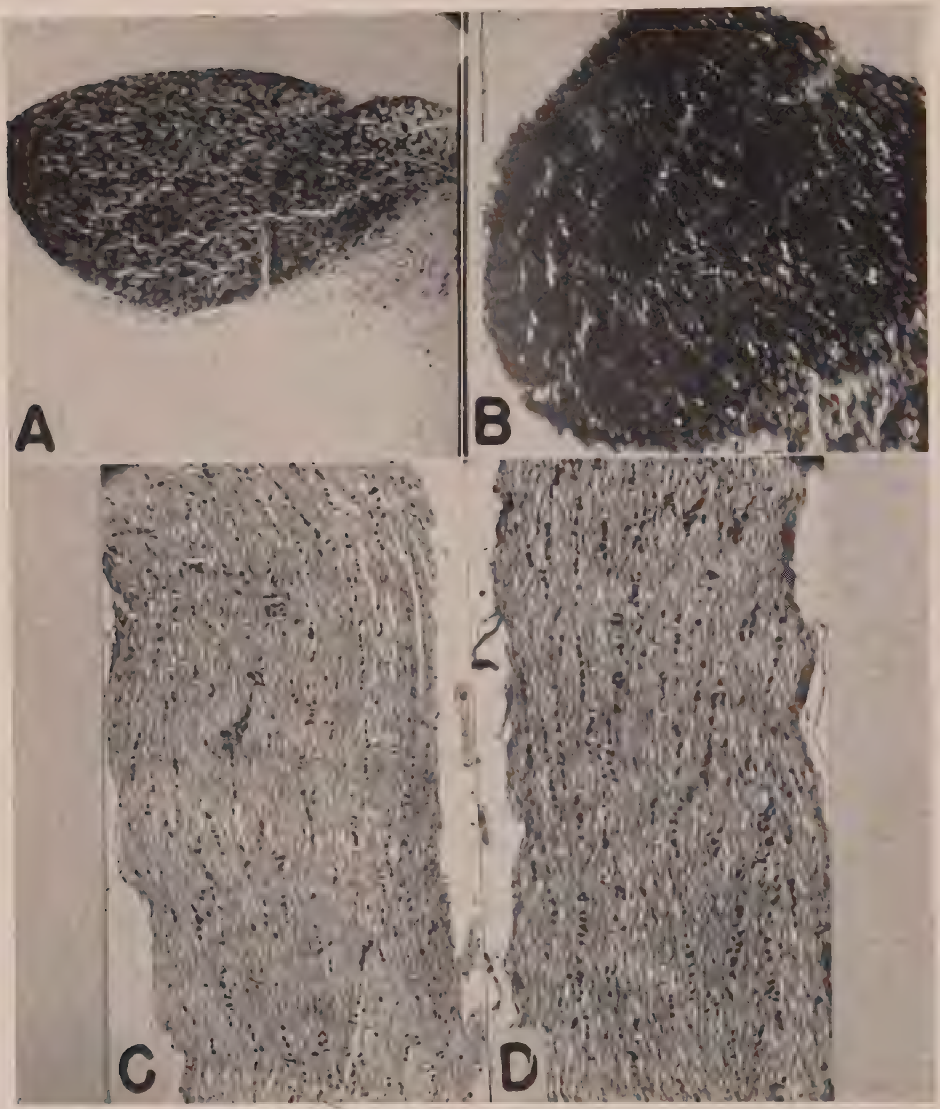

FIG. 1.

A. Optic tract from rat 1-F-3 (exp.); B. Optic tract from rat 1-F-4 (control); C. Optic nerve from rat 2-O-3 (exp.); D. Optic nerve from rat 2-O-6 (control). The optic tracts were stained for myelin by the method of Ulett *et al.*, and the optic nerves were stained by the method of Weil.

ed. The difference between this reading and the zero reading represented the amount of light absorbed by a given section. This procedure was carried out on 6 sections from a given animal. One hundred times optical density was computed for each reading as a measure of relative myelin concentration. The optical density here used was the log of the ratio of incident (galvanometer reading of 80.0) to ensuing light intensity in accordance with the Beer-Lambert law of absorption.

Results. A relative lack of myelin in the optic tracts of underfed rats, as compared to that present in those of litter-mate controls, appeared in the majority of cases. In many instances the difference was of sufficient magnitude to be apparent without the use of the electrophotometer. This is demonstrated by the photomicrographs presented in Fig. 1, in which the optic tracts from Rat 3 (ex-

TABLE I.
Summary and Statistical Evaluation of Results.

Litter	Myelin (experimental)	Myelin (control)	% difference	Significance	% difference body wt	% difference brain wt
Q	35.6	35.9				
	37.6	43.3	8.0	$P = 1$	28.6	13.5
	41.0	45.0		(approx.)		
S	29.9	41.7				
	38.2	43.6	12.6	$P = 1$	64.9	22.0
	28.6	25.4		(approx.)		
Y	42.6	50.2				
	35.1	55.2	19.8	$P = 0.05$	70.7	21.0
	45.3	47.9				
1-F	60.6	63.8				
	32.4	55.9	27.7	$P = 0.15$	57.6	14.9
	37.4	60.4		(approx.)		
2-K	39.8	46.4				
	31.3	40.5	15.9	$P = 0.16$	60.7	17.5
	33.3	37.2		(approx.)		
2-O	29.2	52.2				
	36.7	43.6	34.5	$0.05 > P > .01$	48.3	13.5
	27.0	46.2				
Means	(36.8)	(46.4)	19.8	$P < 0.01$	55.1	17.1

perimental) and Rat 4 (control) of litter 1-F are illustrated (A and B) together with the optic nerves from Rat 3 (experimental) and Rat 6 (control) of litter 2-0 (C and D). The optic nerves were not studied routinely since, in those cases in which they were, those from underfed rats showed the same relative lack of myelin as was apparent in the tracts of the same animals.

Gross examination of the optic foramina of the underfed animals has failed to show any evidence that the amyelination observed in their optic nerves and tracts is due to stenosis of the foramina such as has been described by Moore[4] in carotene-deficient calves.

Statistical analytical methods have been applied to our data by one of us (J.E.R.) and have served to show that the spectrophotometric method is reliable and that the results have statistical significance.

The average values of 6 determinations of relative myelin concentration (100 x optical density) for each underfed and control animal within the respective litters are shown in Table I. The percentage differences given in the table are based upon differences of means between underfed and control rats, relative to the mean of the controls. The succeeding column gives the approximate significance of this difference for each litter based upon analysis of variance within the respective litters. The last 2 columns give average per cent decrease in brain weight and body weight for each litter to indicate relative magnitude of inanition.

An analysis of variance was applied to the data of each litter to determine the sources of variation (1) between the myelin concentrations of underfed and control animals, (2) among the original determinations of myelin concentration, and (3) among the myelin concentrations of underfed and starved animals of each litter. The experimental errors (differences in section thickness, staining, spectrophotometric readings, etc.) were negligible when compared with the two other sources of variation in the experiment, since mean squares ranged from 2 to 7 in all litters except 2-K, where the value was 21. There was, in general, considerable variation between myelin concentrations among animals within the control and experimental groups of each litter; mean squares ranged from 92 to 193 in all litters except 1-F, where the value was 725.

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Comparison of the variance between underfed and control groups, where mean squares ranged from 98 to 2475, with the variance among animals within the groups becomes the critical factor of the analysis within litters. The F-test gave indications of significance as shown in the table. By this test the differences in myelin concentration could have occurred by chance within litters Q and S due to large variance among animals of experimental and control groups, are of moderate degree of significance in litters 1-F and 2-K, and are highly significant in litters Y and 2-O.

In determining the signifiance of underfeeding on optic tract myelination in all 6 litters, 2 major sources of variation, namely, differences in staining between litters and inherent differences in myelin content between litters, were not segregated experimentally. While such inherent variations between litters would be of considerable interest, it appears, from the standpoint of determining the effect of underfeeding on myelination, that the factor of greatest importance is the significance of the differences in myelin density that appeared in the entire group of 6 litters. An F-test comparing total variance between underfed and control animals of each litter with the total variance within litters gave high significance to the difference in myelin ($P < 0.01$). Thus, for the total experiment, there seems to be a significant difference of myelin concentration between control and underfed rats of the order of magnitude of 20%.

Discussion. The relative lack of myelin in the optic nerves and tracts of the majority of our underfed rats, determined photometrically, must be considered as indicative of partial or complete arrest of the process of myelination. Curves constructed from photometric determinations of myelin densities in the optic tracts of successively older members of several litters of albino rats indicate that myelination within the visual pathway continues to be an active process until sometime between the 40th and 50th days as compared with its completion in the medullary pyramid and hypothalamus between the 18th and 30th days.[1] This delay in myelination of the components of the visual pathway may be due to a lack of functional stimulation until after the eyes have opened (14th to 17th day). Tilney and Casamajor[5] reported that they were able to correlate beginning myelination of the optic nerves and the eye-opening reaction in kittens.

The accuracy of the results reported herein depends upon the assumption that the myelin concentration is directly related to optical density in accordance with the Beer-Lambert Law of Absorption. The use of monochromatic light seems to validate this assumption. Whereas slight deviation from the law may be present, the differences found for the entire group of 6 litters appear to be of sufficient magnitude and reliability that such deviation should not alter the general conclusions.

Summary and Conclusions. Quantitative limitation of food intake sufficient to account for average body-weight differences of 55% between underfed and litter-mate control rats, has also resulted in statistically significant differences in myelination of the optic tracts and nerves. The concentration of myelin in the optic tracts of the underfed rats has averaged 20% less than that in the control animals. Since myelination of the optic tracts, as shown by spectrophotometric determinations in litter-mate rats at successively older ages, is not complete until sometime between the 40th and 50th days, the conclusion seems justified that the relative lack of myelin observed in the underfed animals is due to partial or complete arrest of the process of myelination.

The authors wish to thank Mr. W. S. Worley, Supervisor of Special Projects, Product Application Department, Gates Rubber Company, for valuable suggestions and criticisms in regard to the statistical analysis and to acknowledge the painstaking care with which the photomicrographs were prepared by Mr. Glenn Mills.

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16631

Experimental Streptomycin Therapy in Mice.*†

ERNEST JAWETZ.

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In contrast to the sulfonamides, penicillin and streptomycin do not require constant significant blood levels for satisfactory therapeutic results. Experiments in streptococcal infections of mice[1] have clearly indicated that the effect of crude penicillin on the bacterial population outlasts measurable blood levels by many hours. Animals were cured of otherwise fatal infections with single large doses or by spacing injections of penicillin 8-24 hours apart.[2,3,4] Clinical experience in pneumococcal[5,6] and streptococcal[7] disease has also demonstrated the effectiveness of dosage regimens by which aqueous penicillin was injected every 8-12 hours. Experiments with streptomycin have given comparable results.[8]

A possible objection to some of the experimental work presented in this field and its translation into clinical terms is the nature of the laboratory model often used for evaluation of chemotherapeutic effectiveness. Animals are given overwhelming infections, usually by "unnatural" routes, and then treated before there is clinical evidence of the disease. The results thus present the effects of chemoprophylaxis rather than chemotherapy. In the course of studies on a pneumotropic pasteurella of mice[9] observations were made which are of some interest in regard to this question.

Methods. The pneumotropic pasteurella used in this work has been described elsewhere.[9] The organism kills mice only if inoculated intracerebrally (LD_{50}: *ca.* 10^4 bacteria) or intranasally (LD_{50}: *ca.* 5×10^5 bacteria). After intracerebral inoculation neurological signs appear within 12-18 hours and all deaths occur in 36-72 hours with a brain abscess. When infected by the respiratory route the mice show cyanosis and dyspnea, with pulmonary consolidation in 18-24 hours and 70-95% die in 2-6 days. The pasteurella is sensitive to streptomycin. It is regularly completely inhibited by 2-4 γ of streptomycin per milliliter of medium.

The standard inoculum in these experiments consisted of 10^6 bacteria in 0.03 ml of dilutions of a 8-12-hour broth culture, injected intracerebrally or intranasally under light ether anesthesia into 21-day-old (10-14 g) white mice of the stock of the National Institute of Health. The animals were observed for 10 days and deaths recorded. At the end of that period the survivors were autopsied and lung lesions were noted. Among the intracerebrally infected animals there were no untreated survivors, and the treated survivors showed no lesions whatever. The results of these treatment experiments are recorded as the ratio of deaths to the total number of animals in the group. Among intranasally infected animals, however, not all untreated mice died and some of the treated survivors showed extensive lung lesions. In these groups the results are therefore expressed as ratio of "infected" animals to the total number in the group. "Infected"

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* The work described here was performed at the Division of Infectious Diseases, National Institute of Health, Bethesda, Md.

† The author wishes to thank Doctors M. Finland and E. K. Marshall, who read the manuscripts and made valuable suggestions.

[1] Jawetz, E., *Arch. Int. Med.*, 1946, **77**, 1.

[2] Zubrod, C. G., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 400.

[3] Eagle, H., *Am. Int. Med.*, 1948, **28**, 260.

[4] White, H. J., Baker, M. J., and Jackson, E. R., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 199.

[5] Tillet, W. S., Cambier, M. J., and McCormack, J. E., *Bull. N. Y. Acad. Med.*, 1944, **20**, 142.

[6] Marshall, E. K., and Zubrod, C. G., Personal communication.

[7] Jawetz, E., *Arch. Int. Med.*, 1948, in press.

[8] Zubrod, C. G., *Bull. Johns Hopkins Hosp.*, 1948, **82**, 357.

[9] Jawetz, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 46.

TABLE I.
Streptomycin Treatment of Intracerebral Pasteurella Infection.
Mice infected with 100 LD_{50} intracerebrally given 3 subcutaneous injections of 0.1 g/kg streptomycin each, at various intervals.

Treatment time, hr after infection	Deaths/total	% survivors (cured)
2, 6, 24	2/12	83.3
2, 24, 48	1/12	91.6
6, 10, 24	6/12	50.0
6, 24, 48	8/12	33.3
10, 24, 48	12/12	0.0
Controls (untreated)	24/24	0.0

refers to both the dead animals and those which survived but had gross lesions in the lungs.

The streptomycin (a solution with an assay value of 200 mg streptomycin base/ml) was kindly supplied by Dr. H. Welch, Food and Drug Administration, Washington, D.C. Solutions of the required strength were prepared in physiological saline and were injected subcutaneously in amounts of 0.2 ml per dose as indicated in the various dosage schedules.

Results. Table I summarizes selected results of preliminary trials in the treatment of intracerebral infections by subcutaneously administered streptomycin. With fixed individual and total dose the outstanding factor which determined success of therapy was the interval between the injection of bacteria and the first administration of streptomycin. It was immaterial whether the second dose was given 4 or 22 hours after the first. If the total dose exceeded 0.3 g/kg and the first of 3 injections was given not later than 4 hours after intracerebral infection at least 75% of the animals could be cured. For therapy to be successful it had to be started long before neurological symptoms appeared.

When identical treatment schedules were applied to groups of mice infected intracerebrally or intranasally, the milder respiratory infection responded much more readily. With the first dose of antibiotic administered to all mice 4 hours after infection, there was little difference between the results of various dosage schedules (Table II). The ED_{50} (dose resulting in 50% cure)[10] for cure of infection of the central nervous system ranged between 71 and 108 mg/kg with the various regimens, and it was approximately half of those values for the pulmonary disease. These results considered by themselves might indicate similar response of the "artificial" (intracerebral) and "natural" (respiratory) infection to streptomycin treatment. It must be considered, however, that treatment 4 hours after infection is relatively early for the latter and late for the former.

Good evidence, relating the time elapsed before treatment was started to the therapeutic results, was obtained in the comparative tests illustrated in Table III. If treatment was started 4 hours after infection, when neither symptoms nor lesions were present, a number of schedules of therapy were satisfactory. There was no marked difference between total doses of 0.05 g/kg and 0.3 g/kg whether administered in a single dose or in three injections 2 or 24 hours apart. If, however, 18 hours were permitted to elapse between infection and first treatment, statistically significant differences appeared between the results of various low-dose schedules. With a total of 0.1 g/kg, the single dose was quite unsatisfactory, whereas with 3 doses administered at 2-hour intervals, only one-fourth of the animals showed lesions. Three injections 24 hours apart gave intermediate results. These differences between various dosage schedules were not apparent at higher total dose levels (Table III). In order to obtain satisfactory response with long-interval regimens, the dosage had to be larger than with schedules employing injections every 2-4 hours.

Comment. Experimental work in chemotherapy is often based on overwhelming types of infection. Small numbers of highly virulent organisms are introduced into animals in such a manner as to permit rapid multiplication and to produce prompt death. Thus the animals represent little more than living test-tubes to which chemotherapeutic agents are added early in the infection, prior to the appearance of symptoms and lesions. Under

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[10] Quan, S. F., Foster, L. E., Larson, A., and Meyer, K. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **66**, 528.

TABLE II.
Comparison of Treatment Results with Various Dosage Schedules in Intranasal and Intracerebral Pasteurella Infection.
Treatment Schedules.

Total dose streptomycin g/kg	Intranasal infection				Intracerebral infection			
	Single inj.	3 inj. q 2 h	3 inj. q 8 h	3 inj. q 24 h	Single inj.	3 inj. q 2 h	3 inj. q 8 h	3 inj. q 24 h
.05	9/20*	5/20	4/19	5/20	16/20†	14/20	17/20	15/20
.20	4/30	2/30	3/29	4/30	8/20	5/20	2/20	10/20
.30	4/30	1/28	3/30	3/30	7/20	1/20	1/19	6/20
.40	—	2/20	1/18	1/20	—	0/19	0/20	1/20
0	46/50	—	—	—	60/60	—	—	—

All mice infected with 10^6 bacteria. First dose of antibiotic given 4 hr after infection.
* Infected/Total.
† Deaths/Total.

TABLE III.
Influence on Therapeutic Results of the Time Elapsed Before Treatment Was Started.
Total Dose of Streptomycin.*

Treatment started	Dosage schedule	0.1 g/kg		0.3 g/kg		ED_{50} g/kg	S.E. of ED_{50} g/kg
		Inf./Tot.	% lesions	Inf./Tot.	% lesions		
4 hr after infection	Single dose	13/40	32.4	4/10	10.0	.081	± .013
	3 × q2h	8/39	20.5	1/40	2.5	.052	± .009
	3 × q24h	7/40	17.2	3/38	7.9	.063	± .011
18 hr after infection	Single dose	35/40	87.5	12/40	30.0	.219	± .028
	3 × q2h	11/40	27.5	8/40	20.0	.048	± .020
	3 × q24h	19/39	48.6	9/40	22.5	.106	± .014
Controls	0	49/52	94.2	—	—	—	—

All mice infected with 10^6 pasteurella.
ED_{50}: Dose resulting in 50% freedom from lesions. S.E. of ED_{50}: Standard error.[10]
* Only 2 doses are given here. Additional ones were used to calculate ED_{50} and its standard error.

such conditions the natural defense mechanisms of the host do not commonly come into play and the animals die from intoxication or organ necrosis, often without the development of gradually progressive lesions.

In a few human infections, like pneumonic plague, the pathogenesis parallels that seen in experimental animals,[11] and experimental chemotherapeutic results may be directly applicable to patients. In the majority of human infections, however, the disease is much more complex than that of the "test-tube" experiment in animals. Host reactions influence the development of tissue lesions which may have a spontaneous tendency toward healing, and antibiotics probably support mechanism of defense rather than replace them. In human disease chemotherapy is seldom started prior to the development of symptoms or lesions. The extent to which results of "test-tube" experiments in animals mirror events in patients is debatable.

The observations reported in this paper indicate that route of inoculation, nature of disease, and the time treatment is started, can influence considerably the relative merits of various dosage schedules employed in chemotherapy.

Intracerebral pasteurella infection can be cured by streptomycin, subcutaneously administered to mice. This in itself is a remarkable finding. The data cannot be used to support the opinion[12] that some types of human central nervous system infections likewise are cured by intramuscular streptomycin alone. The intracerebral pasteurella infection of mice is strictly of the "test-tube" type and treatment is successful only if started prior to the appearance of symptoms.

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[11] Litchfield, J. T., and Fertig, J. W., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 276.

[12] Hoyne, A. L., and Brown, R. H., *J.A.M.A.*, 1948, **136**, 597.

Mice infected with the same pasteurella by the "natural" respiratory route suffer from a slower, progressive disease and die usually from anoxia due to widespread pneumonic lesions. Streptomycin is effective when started relatively late in that disease process, but even high doses may cure only a certain proportion of the animals. Such late treatment emphasized differences between dosage schedules, not apparent under less critical conditions. The use of similar "natural" types of infection in chemotherapeutic testing is not likely to yield as clear-cut pharmacological results as the "test-tube" types but it might contribute information on the dynamics of antibiotic action important in human disease.

Summary. 1. The therapeutic effect of streptomycin was evaluated in mice infected with a pneumotropic pasteurella either by the respiratory or by the intracerebral route.

2. When treatment was started within 4 hours after infection intracerebrally mice could be saved by streptomycin injected subcutaneously. With a total dose of 0.3 g/kg streptomycin the main factor determining success of therapy was the time interval between infection and first administration of antibiotic.

3. With comparable dosage regimens larger amounts of streptomycin were necessary to cure the intracerebral than the respiratory infection.

4. When treatment of the pulmonary infection was delayed for 18 hours, until symptoms and lesions were present, marked differences appeared in the effectiveness of dosage schedules similar in their effects when treatment was started soon after infection.

5. The possible usefulness of "natural" type infections in chemotherapeutic experiments is discussed and compared to the "test-tube" type of experimental disease.

16632

Failure of an Extract of Pregnant Mares' Urine to Influence Gastric Secretion in Man.

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The effect of extracts of urine upon gastric secretion and upon the course of experimentally induced ulcer has been studied by many investigators. Sandweiss, Saltzstein, and Farbman[1,2] noted delay or prevention of ulcers in the dog following the administration of extracts prepared from human pregnancy urine and the urine from normal women. Various observers[3-9] have reported the presence in human and canine urine of a substance inhibiting gastric motility and the secretion stimulated by histamine and by a meal. Fur-

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[1] Sandweiss, D. J., Saltzstein, H. C., and Farbman, A., *Am. J. Dig. Dis.*, 1938, **5**, 24.

[2] Sandweiss, D. J., Saltzstein, H. C., and Farbman, A., *Am. J. Dig. Dis.*, 1939, **6**, 16.

[3] Friedman, M. H. F., Recknagel, R. O., Sandweiss, D. J., and Patterson, T. L., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 509.

[4] Necheles, H., Hanke, M. E., and Fantl, E., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 618.

[5] Gray, J. S., Wieczorowski, E., and Ivy, A. C., *Science*, 1939, **89**, 489.

[6] Culmer, C. U., Atkinson, A. J., and Ivy, A. C., *Endocrinology*, 1939, **24**, 631.

[7] Gray, J. S., Wieczorowski, E., Culmer, C. U., and Adkinson, J. L., *Am. J. Physiol.*, 1940, **129**, 589.

[8] Culmer, C. U., Gray, J. S., Adkinson, J. L., and Ivy, A. C., *Science*, 1940, **91**, 147.

[9] Friedman, M. H. F., and Sandweiss, D. J., *Am. J. Dig. Dis.*, 1941, **8**, 366.

TABLE I.
Effect of Extract of Pregnant Mares' Urine (Kutrol) on Nocturnal Gastric Secretion of Patients with Peptic Ulcer.

		Control period				After period		
	Case	Vol. (cc)	Free HCl (Cl units)	Total output HCl (mg)	Amt Kutrol (g)	Vol. (cc)	Free HCl (Cl units)	Total output HCl (mg)
				Active Material				
C.O'K. No. 423401 ♂, 43 gastric ulcer	1	585 440	53 50	1120 801	1.125	590	54	1151
J.D. No. 324931 ♂, 50 gastric ulcer	2	619 844	20 22	440 662	1.875	1048	38	1434
K.A. No. 421560 ♀, 44, duod. ulcer	3	919	52	1743	1.875	953	57	1965
A.B. No. 416714 ♂, 44, duod. ulcer	4	637	18	443	1.875	526	24	470
E.D. No. 427655 ♂, 38, duod. ulcer	5	1171	64	2704	7.125	922	51	2207
J.I. No. 214008 ♂, 39, duod. ulcer	6	1326	79	3831	7.125	1252	53	2393
E.R. No. 428179 ♂, 56, duod. ulcer	7	830	68	2062	7.125	647	57	1332
S.R. No. 435305 ♂, 52, duod. ulcer	8	1124	81	3314	7.50	1273	76	3456
				Control Material				
J. McA No. 347846 ♂, 33, duod. ulcer	9	1163	47	2028	7.125	1140	48	2415
A.H. No. 432870 ♀, 56, duod. ulcer	10	918	71	2377	7.125	1262	69	3191
A.H. No. 403706 ♂, 57, duod. ulcer	11	1135	48	1965	7.125	910	53	1785
J.R. No. 232510 ♂, 57, duod. ulcer	12	1054	69	2633	7.125	1139	67	2513

ther study[10-11] resulted in a preparation, designated as urogastrone, free of pyrogenic impurities and gonadotropic hormones. When administered subcutaneously, the quantity of extract effective against the ulcers resulting from the Mann-Williamson operation appeared too small to reduce gastric secretion; the protective action, therefore, was attributed to some factor other than that inhibiting gastric secretion. Sandweiss *et al.*[12] observed beneficial effects against experimental ulcer in the presence of an essentially unaltered acid gastric juice; the protective agent was found in the urine of pregnant and of normal females, but only in small quantities in the urine of patients with peptic ulcer.

The latest preparation to receive attention is an extract of the urine from pregnant mares, termed Kutrol. This material is described[13] as entirely devoid of estrogenic or gonadotropic activity, highly soluble in water, and

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[10] Gray, J. S., Culmer, C. U., Wieczorowski, E., and Adkinson, J. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 225.

[11] Gray, J. S., Wieczorowski, E., and Ivy, A. C., *Am. J. Dig. Dis.*, 1940, **7**, 513.

[12] Sandweiss, D. J., Sugarman, M. H., Friedman, M. H. F., Saltzstein, H. C., and Farbman, A. A., *Am. J. Dig. Dis.*, 1941, **8**, 371.

[13] Sharp, E. A., Personal communication.

manifesting an acceptable degree of antiulcer activity when assayed on the Shay rat; its precise nature is, however, not known. Bercovitz, Page, and Heffner[14] recently have reported encouraging results in the treatment of chronic duodenal ulcer with doses of 0.6 g by mouth daily in divided amounts. It appeared desirable, therefore, to determine the effect of Kutrol upon gastric secretion in man.

Method of Study. The method of investigation was similar to that described previously.[15,16] Twelve patients, 10 with active duodenal ulcer and 2 with gastric ulcer, were studied. Continuous gastric suction was maintained by a Gomco aspirator, the containers being replaced each hour. In 4 patients the 12-hour nocturnal gastric secretion was measured before and approximately 2 hours after the administration of the extract* via the stomach tube; one individual received 0.225 g every 2 hours for 5 doses, a total of 1.125 g; the remaining 3 were given 1.875 g in divided amounts. The subjects were maintained on a liquid diet during the interval when aspiration was discontinued.

In 4 patients gastric suction was continued for 36 hours. After 16 or 17 hours (either at 1:30 or 2:30 p.m.) 7.125 to 7.50 g of Kutrol were introduced into the stomach via the Levine tube. Aspiration was resumed after an interval of 3 hours, and maintained for an additional 16 or 17 hours. The remaining 4 patients were studied in an identical manner but received 7.50 g of an "inactive" material; this group, therefore, served as an additional control. Fluid and electrolyte balance was maintained in the two latter groups by the intravenous administration of 5% glucose in isotonic saline solution.

Results. The data for the 12-hour nocturnal gastric secretion are summarized in Table I. The volume, free acidity and output of hydrochloric acid were not significantly reduced despite the administration of 7.50 g of the extract of pregnant mares' urine, a quantity 12 times larger than the present clinical dose. The control material likewise was without discernible effect. The administration of Kutrol did not decrease the con-

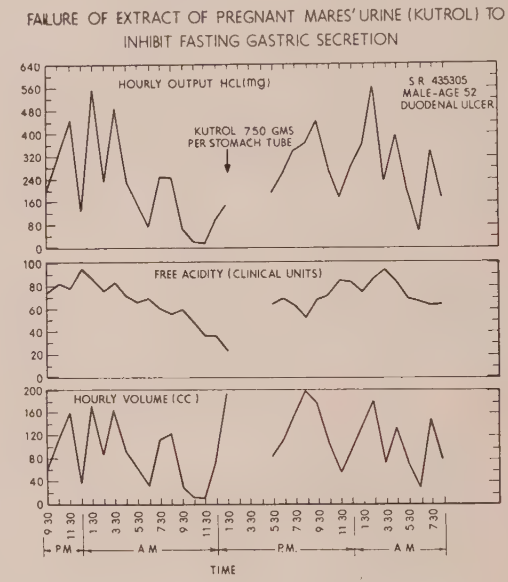

FIG. 1.

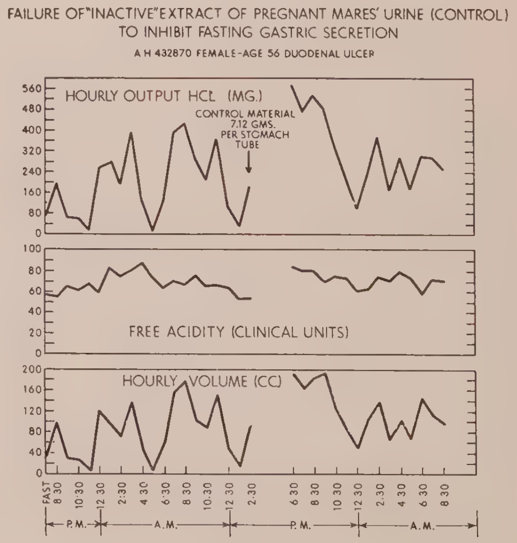

FIG. 2.

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[14] Bercovitz, Z. T., Page, R. C., and Heffner, R. R., Am. Gastroenterological Assn., May 1, 1948, Atlantic City, N. J.

[15] Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Arch. Surg.*, 1948, **46**, 345.

[16] Kirsner, J. B., Levin, E., and Palmer, W. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 90.

* Made available through the kindness of Drs. Z. Bercovitz and E. A. Sharp. The extract was prepared in kapseals, each containing 0.075 g of the active material in the form of a powder.

tinuous, fasting, 36-hour secretion; representative studies with the active extract and the control material are shown in Fig. 1 and 2. The hourly volume, free acidity, and output of hydrochloric acid remained essentially unchanged. No untoward effects were observed in these studies.

Conclusion. The intragastric administration of an extract of pregnant mares' urine, Kutrol, in quantities as large as 7.50 g does not decrease the volume, free acidity, or output of hydrochloric acid in the 12-hour nocturnal gastric secretion or in the continuous 36-hour secretion of patients with peptic ulcer.

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Effect of Forced Expiration upon Finger and Pulse Volume.*

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Although there is general agreement about the peripheral arteriolar constrictive effect of a deep inspiration, the effect of a deep expiration upon peripheral circulation is not yet clarified. Bolton, Carmichael, and Sturup[1] and Gilliat[2] report no effect on finger volume, whereas Uhlenbruck[3] and de Lalla[4] observed a diminution of finger volume, and the latter reported coincident decrease in skin temperature and arterial rate of flow following a forced expiration. Therefore it seemed of interest to study further the possible changes in finger and pulse volume incident to a deep expiration.

Method. Pulse and finger volumes were recorded in two ways using a finger plethysmograph each time: (1) In 11 cases the plethysmograph changes were picked up by a piezo-electric crystal whose output was fed into an amplifier-galvanometer recording system. The deflections of the mirror galvanometer were recorded on film moving at 25 mm per second; in 4 of these tracings a simultaneous ballistocardiogram was recorded. With this method any desired amplification of the pulse volume could be obtained, but the total finger volume changes could not be recorded since the system would not maintain a change of baseline; (2) In 5 cases the output of the plethysmograph was put directly into a Frank capsule whose mirror deflections were recorded in the same manner as above. Although the pulse impulse waves were not large in amplitude with this technic, the total finger volume changes were accurately recorded. Respirations were recorded simultaneously in all cases by a pneumograph-Frank capsule arrangement.

Six normal adult subjects were used, 4 males and 2 females, and a total of 16 records were obtained. They were instructed in the following manner: "At no time take a deep inspiration; at the end of a normal inspiration expire forcefully and rapidly, as much as you can, through your mouth; do not hold your breath, but continue to breathe normally directly afterwards." This maneuver is described in detail since the manner of performance seems to be important.

Results. In all cases there was an immediate decrease in pulse volume, and in the 5 tracings recorded with the Frank capsule there was a simultaneous fall in total finger

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* This work was carried out under a contract between the Office of Naval Research and the University of Rochester, School of Medicine and Dentistry.

[1] Bolton, B., Carmichael, E. A., and Sturup, G., *J. Phys.*, 1946, **86**, 83.

[2] Gilliatt, R. W., *J. Physiol.*, 1948, **107**, 76.

[3] Uhlenbruck, P., *Z. Biol.*, 1924, **80**, 317.

[4] de Lalla, V., *Am. J. Physiol.*, 1948, **152**, 122.

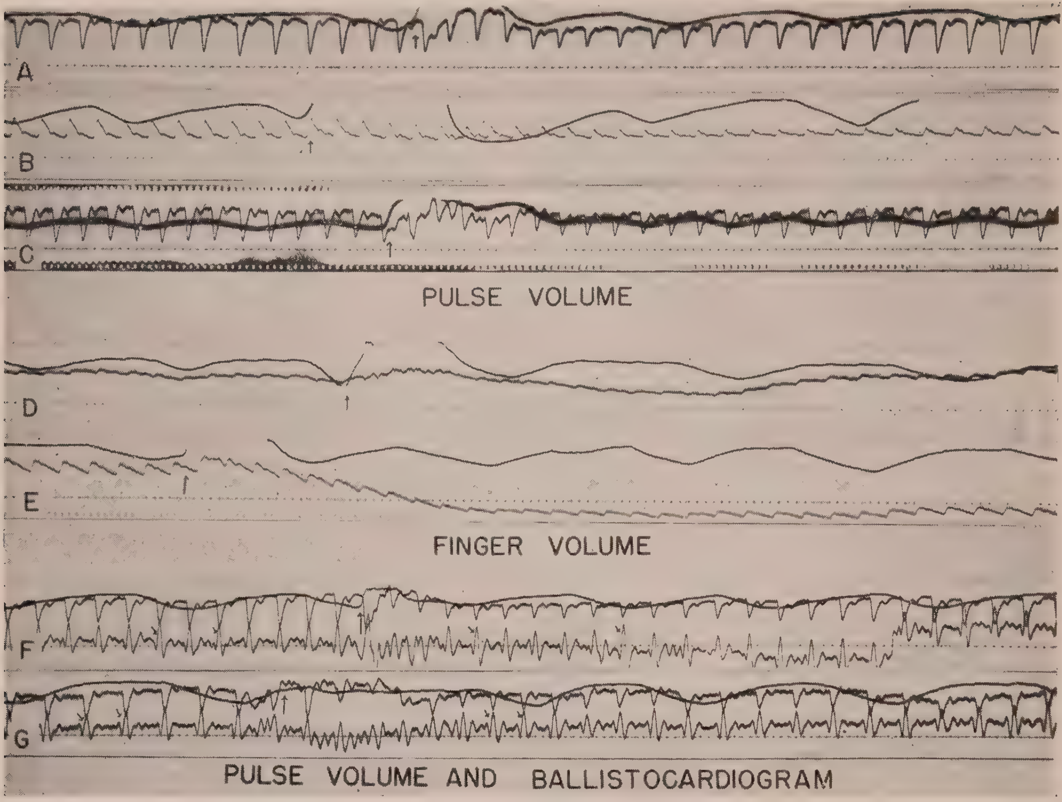

FIG. 1.

Seven records showing the effect of a forced expiration on pulse and finger volume. Respirations are recorded as the top undulating line, with expiration being an upward deflection. The time line is dotted: two-tenths of a second between large dots.

A, B, C are records of finger pulse volume taken with a piezo-electric crystal and do not show baseline changes (see text). Note the immediate decrease in amplitude of the pulse volume stroke in all cases immediately following a deep expiration (arrows). The recovery in B occurred in 16 seconds and is not shown. See Fig. 2 for graphs.

D and E were taken with a Frank capsule, and recorded total finger volume changes as well as individual pulse volumes. Note the fall in total finger volume and the decrease in pulse volume following a deep expiration (arrows). The recovery in E occurred within 30 seconds and is not shown.

F and G are records of finger pulse volume taken as in A with a simultaneous ballistocardiogram recorded below. Note that although the pulse volume diminishes directly after a deep expiration (large arrow), the IJ stroke of the ballistocardiogram (small arrows) does not. See Fig. 3 for graphs and text for interpretation.

volume (Fig. 1, 2). The average duration of effect was 16 seconds for diminished pulse volume and 24 seconds for the diminution of total finger volume. The individual response of pulse volume change can be determined from Fig. 2. The individual duration of total finger volume diminution varied from 10 to 40 seconds.

In the 4 cases where the ballistocardiogram was recorded with the pulse volume, the height of the IJ stroke of the ballistocardiogram in millimeters and the height of the pulse volume stroke in millimeters was averaged for the control period in each case. The deviation from the respective control average of the height of each IJ stroke and pulse volume stroke in millimeters was plotted against the corresponding beat. These correlation graphs are shown in Fig. 3 and representative tracings are given in Fig. 1F and G. In no case was the total stroke volume decreased during or after the deep expiration, whereas the pulse volume had immediately decreased, and remained so even

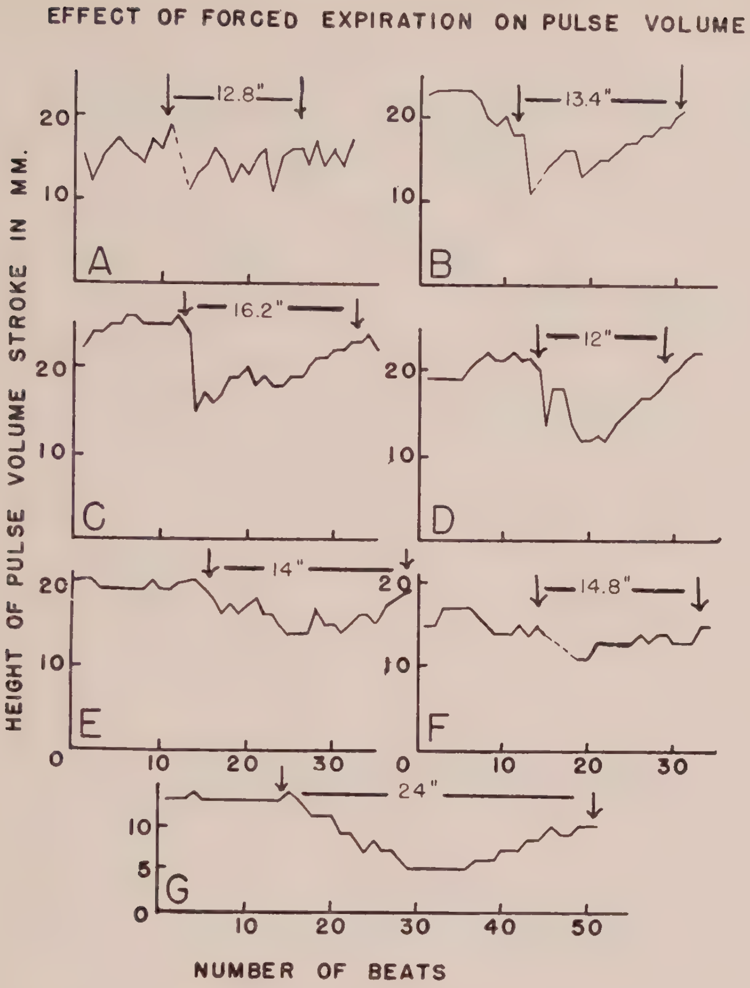

Fig. 2.

Graphs of the effect of a deep expiration on finger pulse volume in 7 cases. The height of the pulse volume stroke (recorded as in Fig. 1A) measured in millimeters is plotted against the corresponding beat. Note the immediate drop in pulse volume in all cases following a deep expiration (first arrow). The recovery time in seconds is indicated between the first and second arrows, in each case. The dotted lines in A, B, and F represent strokes which were not measurable because of artifacts caused by the motion of the subject.

though the corresponding ballistocardiogram beat was not smaller than a comparable control beat.

Discussion. A deep, forced expiration performed in the manner described produced a decrease in both total finger volume and purse volume, in the 6 subjects studied. This confirms the reports of Uhlenbruck and de Lalla.

By using the height of the IJ stroke as a measure of relative stroke volume[5,6] the simultaneous ballistocardiogram may be interpreted as confirmatory evidence for reflex vasoconstriction since the only other probable cause for the observed diminution of pulse and total finger volume (*i.e.* diminished left ven-

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[5] Starr, I., Rawson, A., Schroeder, H., Joseph, N., *Am. J. Physiol.*, 1939, **127**, 1.

[6] Brown, H. R., Jr., and de Lalla, V., Jr., Unpublished data.

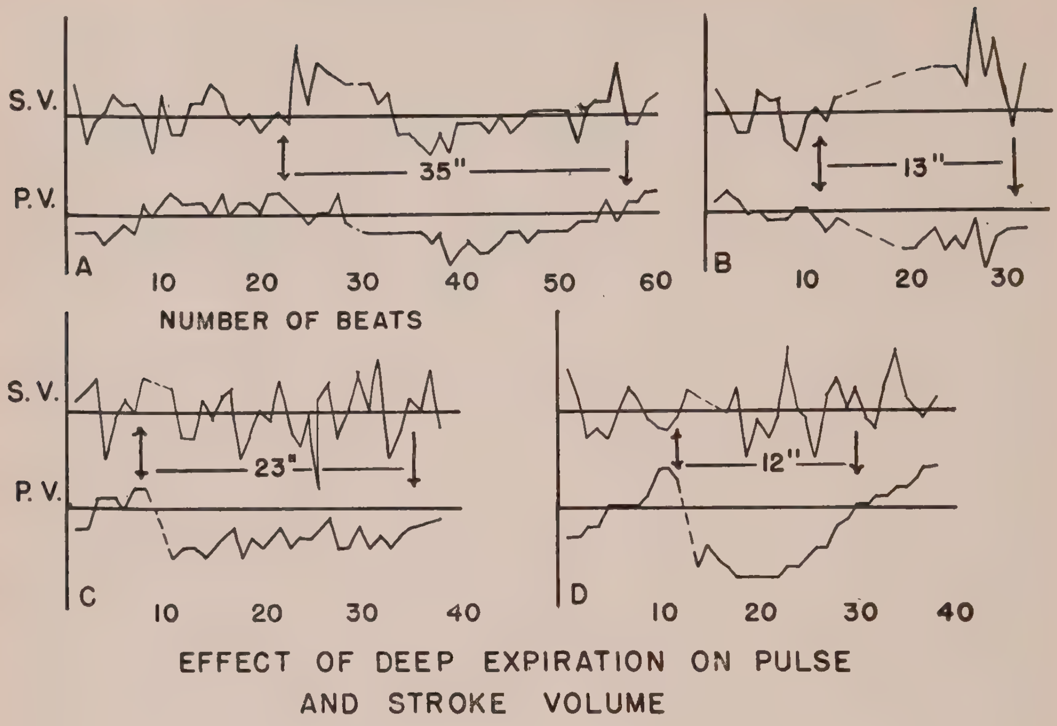

FIG. 3.

Four graphs of the effect of a deep expiration on the height of the finger pulse volume stroke and on the height of the IJ stroke of the ballistocardiogram (relative total stroke volume). The deviation in millimeters from the control average height of pulse volume and IJ stroke in millimeters is plotted against the corresponding beat in each case. Note that although the pulse volume curve (P.V.) drops after a deep expiration (first arrow), the relative stroke volume curve (S.V.) does not change significantly. The pulse volume recovery time is indicated in seconds between the first and second arrows in each case. The large swings of the S.V. curve reflect the respiratory variation of the total stroke volume. See Fig. 1F and G for representative tracings. The dotted lines represent strokes which were not measurable because of artifacts caused by the motion of the subject.

tricular output) does not seem to have occurred. (See Fig. 1F, G and 3). Since the IJ stroke reflects total stroke volume, and since the right ventricular stroke volume is known to decrease in expiration[6–8], then one might expect to find smaller IJ strokes during or after the forced expiration than in comparable control beats, if the left ventricular stroke volume had diminished or remained the same. That this was not observed indicates that the left heart stroke volume was increased during the forced expiration at the same time that the pulse volume was observed to have decreased. This deduction is sound in view of the report of Shuler *et al.*, which states that the left ventricular stroke volume increases with expiration.[7] These findings suggest that a reflex vasoconstriction is the cause of the diminution of pulse and total finger volume incident to a forced expiration.

One of us had already postulated that stretch receptors in the large extrathoracic veins may be the origin of this reflex.[4] However, it is not necessary that these receptors be located in the large veins themselves in order to respond to increased abdominal venous pressure. Gammon and Bronk[9] have demonstrated Pacinian corpuscles in the mesentery which respond to engorgement of the neighboring tissue by raising the systemic blood pressure (*i.e.* arterial vasoconstriction). They also showed that these receptors would respond in the same manner to increased pressure in the abdominal vena cava. It is possible that these receptors are the initiators of the observed reflex.

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[7] Shuler, R., Ensor, C., Gunning, R., and Moss, W., *Am. J. Physiol.*, 1942, **137**, 602.

[8] Lauson, H. D., Bloomfield, R. A., and Cournand, A., *Am. J. Med.*, 1946, **1**, 315.

[9] Gammon, G. D., and Bronk, D. W., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 788.

Should this be true, then it is understandable why the expiration may not produce diminution of pulse and finger volume if not forced. It is likely that a critical intrathoracic pressure must be reached before the abdominal venous pressure is raised enough to stimulate the receptors. In that event, a deep expiration that does not do this will not elicit the reflex.

Summary. 1. Twelve records of the effect of a deep, forced expiration on finger pulse volume were obtained from six subjects. In four of these records a ballistocardiogram was recorded simultaneously. In 5 other tracings total finger volume changes were recorded.

2. In all cases the pulse volume and total finger volume (when recorded) decreased immediately following the deep forced expiration. Average duration of diminution was 16 seconds for pulse volume and 24 seconds for total finger volume.

3. The absence of a simultaneous decrease in corresponding ballistocardiogram beats suggests that the observed finger changes were due to reflex arteriolar constriction, possibly initiated by abdominal presso-receptors in the great veins or mesentery.

Appreciation is expressed to Prof. William S. McCann for his advice and criticism in the review of the manuscript, and to Prof. W. O. Fenn for his helpful suggestions during the course of the work.

16634

Location of Communications between Cognate Bed of Descending Ramus of Left Coronary and Adjacent Collateral Vascular Beds.*

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More than 40 years ago, Hirsch and Spalteholz[1] noticed that after ligation of a coronary artery, the area of infarction was much smaller than the myocardium apparently supplied by that artery (cognate bed). Two years later Miller and Mathews[2] made the same observations, and suggested that this was due to the presence of fine epicardial arterial anastomoses. Since that time, numerous additional reports have appeared describing the variability in the occurrence of and in the size of infarcts produced by coronary artery ligation.[3-8] Sutton and Davis[9] suggested tying the superficial anastomotic vessels in order to get a large infarct.

No actual studies, however, appear to have been done in order to demonstrate whether or not the blood supplied by the superficial

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* Supported by a grant from the Life Insurance Medical Research Fund.

[1] Hirsch, C., and Spalteholz, W., quoted by L. Gross, 1921, Hoeber, N. Y.

[2] Miller, J. L., and Mathews, S. A., *Arch. Int. Med.*, 1909, **3**, 476.

[3] Smith, F. M., *Arch. Int. Med.*, 1918, **22**, 8.

[4] Mendlowitz, M., Schauer, G., and Gross, L., *Am. Heart J.*, 1937, **13**, 663.

[5] Karsner, H. T., and Dwyer, J. F., *J. Med. Research*, 1916, **34**, 21.

[6] Smith, F. M., *Am. J. Med. Sc.*, 1918, **156**, 706.

[7] Gold, H., Travell, J., and Modell, W., *Am. Heart J.*, 1937, **14**, 284.

[8] O'Neill, J. F., and Thomas, W. C., Personal communications, 1947.

[9] Sutton, D. C., and Davis, M. D., *Arch. Int. Med.*, 1931, **48**, 1118.

vessels is the cause of the variability in the resulting infarctions. It was reasoned that if the principal collateral supply is through the superficial vessels, then if an artery were ligated and all superficial anastomotic branches were interrupted, an infarct of fairly predictable size should result. On the other hand, if a consistent infarct was not obtained, then the collateral blood supply must come by way of deep communicating vessels. This was tested by the following procedure:

Method. Mongrel dogs of both sexes and weighing 5 to 16 kg were anesthetized with intravenous sodium pentobarbital† (40 mg/kg), artificial respiration was administered and aseptic thoracotomies performed by subperiosteal rib resection.

Immediately before operation the dogs were given 10,000 units of penicillin. Postoperatively they received a total of 90,000 units in divided doses 3 times a day for 3 days. In the last 24 thoracotomies penicillin in emulsified oil (Pendil‡) was used to maintain the penicillin level through the 2 first postoperative nights with water soluble penicillin being given in the day. This materially reduced the number of injections required and was found to be much more convenient. In these series totalling 52 thoracotomies treated with penicillin there was not a single death from infection nor did any show more infection than a drop of pus around an unremoved skin suture. This is in marked contrast to Miller and Mathews'[2] results of a death rate of 60% due to sepsis.

Three groups of procedures were carried out upon the hearts.

Group A. In 23 dogs the anterior descending arteries and the accompanying veins were ligated 1 to 2 cm below the bifurcation of the left coronary arteries. This is the region where there is usually a relatively large branch going to the left ventricular myocardium. In 11 of these dogs the main vessel and this large branch were individually tied; in 9 dogs the ligature was above the first large branch; in 3 dogs the ligature was below the first main branch which was left intact (see diagram). The areas of distribution of the arteries were exposed and the edges touched with a hot iron cautery producing burns light brown in color and about 3-5 mm in width. Similar burns on excised and living hearts showed that burns of this type extend into the myocardium less than a millimeter. The chests were closed in layers (Fig. 1).

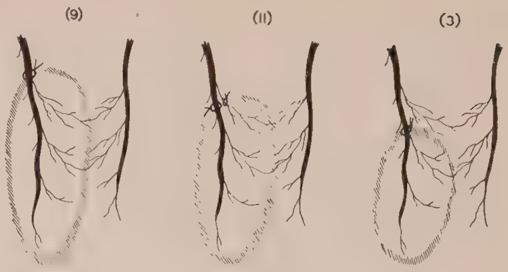

FIG. 1.

Diagram of the site of ligation of the descending ramus of the left coronary artery with the site of the superficial burn indicated by crosshatching. The figures at the top of the diagram indicate the number of animals receiving each type of ligation plus cautery. All of the above types are included in Group A.

Group B. In 8 animals a ligation was done as described for the first 20 of Group A but cauterization was omitted.

Group C. In 4 animals cauterization as described was done without ligation.

All animals were sacrificed 8 to 14 days after operation.

Results. Table I. Six animals died within 24 hours after the ligation-cauterization procedure. The cause of death was probably ventricular fibrillation since no other cause could be found at autopsy. These animals were dropped from the series because their early deaths did not allow enough time for the development of gross evidence of infarction. Recently preoperative and postoperative intravenous procaine, 10 mg/kg at each administration, was added to the procedure. Of 6 dogs so treated, one died of fibrillation while the chest was still open. The remainder survived until sacrificed. The latter death casts some doubt on the efficacy of procaine in preventing the fibrillation which occasionally accompanies this procedure.

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† Supplied by Premo Pharmaceutical Labs, Inc., New York City, N. Y.

‡ Supplied by Endo Products, Inc., Richmond Hill, N. Y.

TABLE I.
Results in Animals Sacrificed.

Groups	Gross infarction	No gross infarction	No. in group
Group A	17	0	17
” B	6	2	8
” C	0	4	4
Total in Groups A and B	23	2	25

The difference in occurrence of infarction was significant below the 5% level. In this case:

$$X^2 = \frac{(ad - bc)^2 N}{(a + b)\ (c + d)\ (a + c)\ (b + d)} = 4.619^{10}$$

The results on the remaining animals were as follows: *Group A.* Cautery and Ligation. All 17 of the hearts of the animals surviving the first 24 hours showed definite gross infarctions involving the full thickness of the myocardium and extending to the burned areas.

Group B. Ligation. Two of these showed extensive infarctions approximating those seen in Group A; 4 showed infarctions smaller than area apparently supplied by the artery; 2 showed no gross evidence of infarction. All areas of necrosis stopped abruptly at the right edge of the anterior vessels, *i.e.*, the necrosis did not involve the right ventricular myocardium. These were different than the ligated cauterized infarctions where the necrosis extended to the cauterized area on the right ventricle. None of the infarctions in either series involved the interventricular septum.

Group C. The only lesions found were adhesions between the burned area and the pericardium. None showed gross infarctions.

Discussion. The X^2 value of 4.619[10] comparing incidence of infarction in Groups A and B, shows that the probability of chance occurrence lies well below the 5% level. Therefore, we consider this difference to be probably significant.

Lowe[11] and Lowe and Wartman[12] have shown how branches from the epicardial vessels go to their respective layers of the myocardium. It appears possible from our studies that these small arteries may be end arteries, in the strict sense, and have only capillary and small arteriolar anastomoses with the other arteries supplying the same layers. The present study suggests that the deep small anastomosing vessels cannot prevent necrosis of a very large portion of myocardium when its epicardial supplying arteries are interrupted.

Summary and conclusion. The frequent failure to obtain infarcts and the variability in the size of the infarcts which follow ligation of a coronary artery in the dog suggest the presence of prominent anastomotic communications with collateral vascular beds. To determine whether these communications were in deep or superficial vessels, the consistency of infarction with simple coronary ligation was compared with the infarction which resulted from coronary ligation plus occlusion of the superficial vessels by cauterization about the cognate bed of the ligated vessels. Since the latter procedure produced infarctions of predictable size in all of the 17 dogs so treated, which survived for 24 hours or longer, it is concluded that the principal anastomotic communications with collateral vessels lie in the epicardium.

It is suggested that ligation plus superficial cauterization may be of definite value in providing a fairly well standardized procedure for the experimental study of myocardial infarction.

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[10] Chambers, E. G., Cambridge Univ. Press, 1940, Cambridge, England.

[11] Lowe, T. E., *Am. Heart J.*, 1941, **21**, 326.

[12] Lowe, T. E., and Wartman, W. B., *Brit. Heart J.*, 1944, **6**, 115.

16635

Effect of Hypophyseal Growth Hormone upon the Urinary Nitrogen Excretion of Fasting Rats.*

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It was shown by Harrison and Long[1] that the administration of a saline extract of the anterior pituitary would decrease the urinary nitrogen excretion of fasting rats. It seemed of interest to determine if pure growth hormone would produce the same results.

Methods. The hormone used was prepared according to the previously published method.[2] All animals used were "plateued" female rats of the Long-Evans strain which weighed between 150 and 200 g and were from 5-8 months of age. There were 2 groups of 5 animals each and during the control periods each animal was fed 12 g of the stock diet[†] daily, this amount being eaten completely. The animals were kept in individual metabolism cages; urine collections were made daily; and the urine was analyzed by the micro-Kjeldahl procedure for its total nitrogen content.

Following a 5-day preinjection control period, all animals were fasted for 2 days and the animals in Group 1 were given intraperitoneally 200 γ of growth hormone per day. (The dose was divided so that the hormone was given in 100 γ doses each morning and evening.) The animals in Group 2 served as the uninjected controls. At the end of the 2-day fasting period, hormone administration was stopped and the animals were again fed 12 g of diet per day. At the end of a second control period of 10 days the rats were again fasted and hormone administration again started. During this injection period the animals in group 2 received the same dose of hormone as above, while the previously injected animals served as the controls. The fast was continued for 5 days, 2 of the control rats dying on the 5th day. Growth hormone injections were continued for 6 days after termination of the fast. Urinary nitrogen excretion was then determined for a subsequent 9-day post-injection control period.

Results and Comment. The data of the experiment are presented graphically in Fig. 1 in which the average daily nitrogen excretions of each group are plotted. It will be seen that the average nitrogen excretion of the growth hormone-treated animals was lower than that of the controls during each fasting period. The average nitrogen excretions of the injected animals during the first and second fasting periods were 91 and 64 mg per day respectively as compared to 116 and 126 mg per day excreted by the controls during the same periods. These were compared statistically according to the methods of Fisher[3] and a p value of 0.02 and of less than 0.01 were obtained for the first and second fasting periods respectively.

It is also of interest to note that following the re-institution of feeding after the second fasting period the nitrogen excretion of the animals receiving growth hormone remained

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* Aided by grants from the American Cancer Society (through the National Research Council, Committee on Growth), the National Institute of Health RG 409, and The Research Board, University of California.

[1] Harrison, H. C., and Long, C. N. H., *Endocrinology*, 1940, **26**, 971.

[2] Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1945, **159**, 353.

[†] The diet fed consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder 10.0%, NaCl 0.75%, $CaCO_3$ 1.5%, hydrogenated vegetable oil (Crisco or Primex) 5.25%. To each kg of diet were added 3.5 g of Sardilene (fish oil concentrate containing 3000 USP units of vitamin A, and 400 chick units vitamin D per g).

[3] Fisher, R. A., Statistical Methods for Research Workers, 10th Ed., Edinburgh, London, Oliver and Boyd (1946).

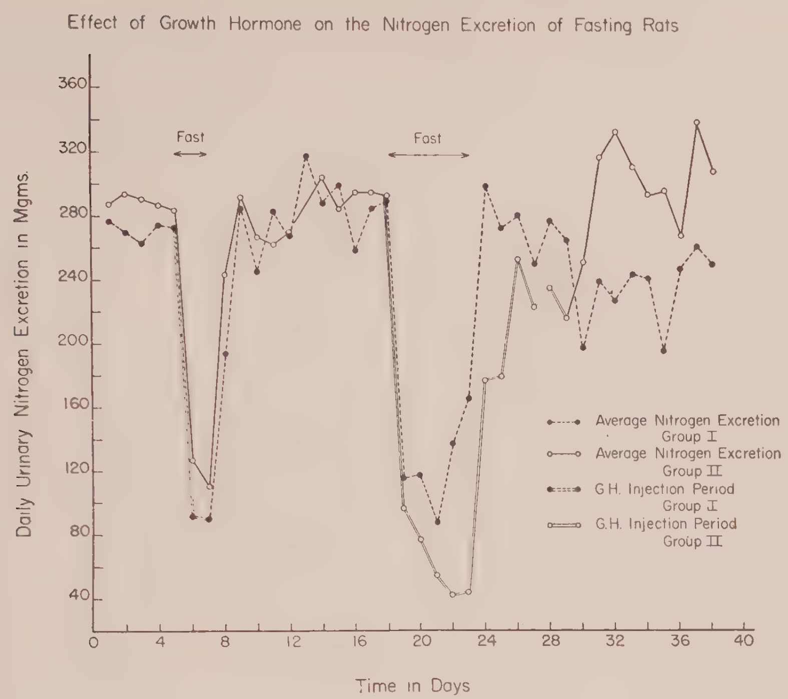

FIG. 1.

below that of the controls. Following the cessation of growth hormone administration there was an increase in nitrogen excretion comparable to that previously described.[4] The 2 animals in the control group that died showed the typical pre-mortal rise in nitrogen excretion while none of the treated animals showed a similar rise during fasting.

That growth hormone has a protein anabolic effect in fed animals is well shown by the fact that it produces general body growth and nitrogen retention.[4] That it reduces urinary nitrogen excretion in fasting rats is evidence that its *net* effect is to reduce protein catabolism under these conditions. However, this type of data does not permit any critical evaluation of the mechanism of action of growth hormone on protein metabolism.

Conclusion. Growth hormone at a dose level of 200 γ per day reduces the urinary nitrogen excretion of fasting adult female rats.

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[4] Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M., *Endocrinology*, 1948, **42**, 153.

16636 P

Relationship of the Pituitary to the X zone of the Mouse Adrenal.*

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The relationship of the X zone of the mouse adrenal[1-3] to the pituitary is not known. It was considered that the use of the technic of hypophysectomy might produce results of help in the elucidation of this problem.

Material and Methods. Virgin female "Swiss albino" mice, aged 40-42 days at the commencement of the experiment, were used. The adrenal X zone is well developed at this age. Hypophysectomy was performed by the parapharyngeal approach. Injection of the hormones noted below was started 24 hours after the operation (except that the chorionic gonadotropin injections were commenced 48 hours after operation) and the animals killed 24 hours after the last injection. The mice were grouped as follows:

1. Twelve intact untreated animals killed at 54-58 days of age.
2. Eleven animals hypophysectomised for 12 days and 8 for 14 days and then killed.
3. Nine hypophysectomised animals injected with a pituitary preparation, principally adrenocorticotropin‡ in nature, 1 mg per day divided into three intraperitoneal injections, for 12 days.
4. Nine hypophysectomised animals injected subcutaneously with 1 mg of pituitary gonadotropin, principally LH‡ in nature, for 10 days.
5. Five hypophysectomised animals injected subcutaneously with 1 mg per day of pituitary gonadotropin, principally FSH‡ in nature, for 10 days.
6. Six hypophysectomised animals injected intraperitoneally with 11 I.U of human chorionic gonadotropin ("Korotrin") per day for 10 days.

The right adrenal was treated routinely after fixation in Bouin's and stained in Harris' haemotoxylin and eosin or Heidenhain's Azan. The left adrenals were fixed for histochemical studies and are not being reported upon here. The thymus, uterus and ovaries were weighed; the ovaries were then treated as for the right adrenal. The completeness of hypophysectomy was judged by the examination of serial sections of the appropriate area in 2 cases from each group; in the remainder the success of the operation was judged by the examination of the sella turcica region under binoculars (x 15). Added confirmation was given by the weight of the animal and, where relevant, by the weights of the uterus and ovaries and the histological condition of the ovaries and adrenals.

Results. The intact untreated animals showed a well-developed X zone with deep eosinophilia of the cytoplasm and large vesicular basophil nuclei. After 12 to 14 days of hypophysectomy the X zone became more compact,§ the cytoplasm of the cells had

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* Aided in part by a grant from the Commonwealth Fund.

† Fellow of the Commonwealth Fund, on leave of absence from the Department of Zoology, Liverpool University.

[1] Howard-Miller, E., *Am. J. Anat.*, 1927, **40**, 25.

[2] Parkes, A. S., *Phys. Rev.*, 1945, **25**, 203.

[3] Jones, I. Chester, *Q.J.M.S.*, 1948, **89**, 53.

‡ The author is very grateful to the Armour Company for the ACTH and LH preparations and to the Schering Corporation for the FSH. The ACTH used was lot 42B about 36% potency of the Armour La-1-A standard; see Forsham, P. H., *et al.*, *J. Clin. End.*, 1948, **8**, 15, for discussion of this and of the type of contaminants. The LH type of gonadotropin was Lot FW-234, the main contaminants being posterior lobe hormones; there was slight FSH activity. The FSH type of gonadotropin ("Synergist") produced good follicle development in the ovaries of hypophysectomized mice with some luteinization.

§ Leblond, C. P., and Nelson, W. O., *(Bull. Hist. Appl.*, 1937, **14**, 181), note the disappearance of the X zone after hypophysectomy but their data include only four mice which would have had an X zone at the time of operation and the length of time for which these were hypophysectomized is not made clear. The "brown degeneration" occurring in another series[4] did not appear in the hypophysectomized animals in this experiment.

shrunk and stained only faintly with eosin; the nuclei were irregular and stained deeply with haemotoxylin (remains of the X zone can be seen up to 100 days after hypophysectomy[4]). The zona fasciculata showed the atrophy characteristic of the adrenals of other animals after hypophysectomy;[5] the zona glomerulosa showed no change in the routine histological preparations. In the ACTH injected animals the X zone was unaffected but the rest of the cortex resembled that of the normal animal. In the LH injected animals the X zone was maintained in a condition similar to that of the normal animal; the zona fasciculata was unaffected and showed the atrophy characteristically appearing after hypophysectomy; the zona glomerulosa was not affected. In the FSH injected animals the X zone had not degenerated as far as in the hypophysectomised controls but the degree of maintenance was much less than in the LH injected animals; the rest of the cortex had an appearance typical of that of the hypophysectomised animal. In the animals injected with chorionic gonadotropin the X zone had disappeared and a medullary connective tissue capsule had formed; the remainder of the cortex was unaffected by the hormone.

Discussion. The X zone depends upon the pituitary for the maintenance of its normal histological structure and a pituitary gonadotropin seems to be the maintaining factor. This gonadotropin is possibly LH, the effect of the FSH preparation then being due to the contaminating LH. The rich LH secretion of castrate animals[6,7] would account for the persistence and growth of the X zone of the prepuberally castrated male mouse and for the evocation of a "secondary X zone" in the mouse castrated when adult.

The chorionic gonadotropin, which has some physiological properties in common with LH, swept the X zone away, paralleling its action in the intact animal.[8] It is equivalent in its results to the direct action of testosterone on the X zone of the *hypophysectomised* prepuberally castrated male mouse.[9] In normal animals, then, it may be that the maintaining factor of the pituitary is overridden, in the male at puberty by the direct action of androgens on the X zone and in the female early in first pregnancy by an "anterior pituitary-like" hormone arising from the conceptus or the endometrium.[10] The X zone seems to be independent of ACTH which indeed may be concerned mainly with the zona fasciculata.[5] The results require confirmation by the use of chemically pure pituitary factors when these are available.

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[4] Jones, I. Chester, *Anat. Rec.*, 1948, **100**, (4) Supp., p. 49 and unpublished.

[5] Deane, H. W., and Greep, R. O., *Am. J. Anat.*, 1946, **79**, 117.

[6] Hellbaum, A. A., and Greep, R. O., *Am. J. Anat.*, 1940, **67**, 287.

[7] Greep, R. O., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 214.

[8] Takewaki, K., *J. Facult. Sci.*, Tokyo, 1935, **4**, 83.

[9] Jones, I. Chester, 1948, in preparation.

[10] Rowlands, I. W., *J. Endocrinology*, 1947, **5** (3), Supp. p. xx.

16637

Deleterious Effects of Pancreas in the Hyperthyroid Rat.*

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In preliminary experiments on the effects of feeding various tissues on symptoms of thyrotoxicosis in the rat, it was observed that immature rats fed diets containing both desiccated thyroid and pancreas lived but a fraction of the time of animals fed similar diets with either thyroid or pancreas omitted. The present experiment was undertaken to obtain further data on this interrelationship.

Procedure and Results. Four basal rations were employed in the present experiment: diets A, B, C, and D. Diet A was a synthetic ration. Diets B, C, and D were similar in composition but contained 10% pancreas, activated pancreas† or papain, added in place of an equal amount of sucrose. All 4 rations were supplemented with 0.0 and 5.0 g of U.S.P. desiccated thyroid per kg of diet. Sixty-four female rats of the Long-Evans strain were selected at 21 to 23 days of age and an average weight of 45.4 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and were fed *ad lib.* the diets listed in Table I. Feeding was continued for 28 days or until death, whichever occurred sooner.

Results are summarized in Table II. Findings indicate that immature female rats fed diets containing both pancreas and desiccated thyroid lived but a fraction of the time of animals fed similar diets with either pancreas or thyroid omitted. On diet A_1 100% of the rats survived the experimental period of 28 days. On diets B_1 and C_1 (containing pancreas and activated pancreas respectively) the mortality rate was 100% with an average survival time of 15.5 and 7.4 days respectively. Findings with papain (diet D_1) were substantially the same as those with pancreas preparations. A marked retardation in growth was observed in animals fed diets B_1, C_1 and D_1. In the activated pancreas series (diet C_1) 9 of the 10 rats in this group lost weight during the first week of feeding. On diets B_1 and D_1 the average gain in weight during the first week of the experiment was less than 10 g in contrast to an average gain of 24.7 g on diet A_1 and approximately 20 g for rats fed control rations (diet A_2, B_2, C_2 and D_2). No significant difference in growth was observed on the 4 diets employed in the absence of dietary thyroid, although gain in body weight was somewhat greater on the synthetic ration (diet A_2) than on diets containing pancreas or papain. All rats fed thyroid-free rations survived the experimental period of 28 days.

No data are available concerning the cause of death in animals fed diets containing desiccated thyroid and either pancreas or papain. Since both of the latter substances contain proteolytic enzymes and since activated pancreas (in which the trypsinogen is converted into trypsin) was more active than the ordinary desiccated material, it was felt that the deleterious effects of pancreas or papain in the thyroid-fed rat may have been due to

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* This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 216 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army. Contribution No. 193 from the Department of Biochemistry and Nutrition, University of Southern California.

† Activated pancreas is desiccated pancreas whose trypsinogen has been converted into trypsin. We are indebted to Mr. Ezra Levin of the VioBin Corporation, Monticello, Ill., for these preparations.

[1] Sure, B., *J. Nutrition*, 1941, **22**, 499.

TABLE I.
Composition of Experimental Diets.

Dietary component	Diet A_1 and A_2	Diet B_1 and B_2	Diet C_1 and C_2	Diet D_1 and D_2
Pancreas*	0	10	0	0
Activated pancreas†	0	0	10	0
Papain‡	0	0	0	10
Casein§	22	22	22	22
Salt mixture‖	4.5	4.5	4.5	4.5
Sucrose	73.5	63.5	63.5	63.5

To each kg of the above diets were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 10 mg, and choline chloride 1.2 g. Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg, and a vitamin A-D concentrate¶ containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

U.S.P. desiccated thyroid (Armour) was incorporated in diets A_1, B_1, C_1, and D_1 at a level of 5.0 g per kg of diet, replacing an equal amount of sucrose.

* Desiccated Pancreas, VioBin Corporation, Monticello, Ill.

† Activated Pancreas, VioBin Corporation, Monticello, Ill.

‡ Frenco's Papain Absolute—Powder, Frenco Laboratories, Nogales, Ariz.

§ Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‖ Sure's Salt Mixture No. 1.[1]

¶ Nopco Fish Oil Concentrate assaying 800,000 U.S.P. units of vit. A and 80,000 U.S.P. units of vit. D per g.

TABLE II.
Effects of Pancreas Feeding on the Survival Time of Hyperthyroid Rats.

Dietary group	No. of animals	Initial wt (g)	Gain in body wt 28-day period* (g)	% surviving†	Avg survival time of decedents* (days)
			Desiccated thyroid series.		
A^1	10	45.1	65.9 ± 4.6 (10)	100	—
B_1	10	45.2	— —	0	15.5 ± 1.7
C_1	10	45.4	— —	0	7.4 ± 0.6
D_1	10	45.2	25.0 ± 4.3 (2)	20	18.6 ± 1.2
			Control series.		
A_2	6	45.5	91.8 ± 4.5 (6)	100	—
B_2	6	45.2	77.1 ± 3.1 (6)	100	—
C_2	6	45.0	76.3 ± 2.8 (6)	100	—
D_2	6	45.5	73.7 ± 4.7 (6)	100	—
			Thyroxin series.		
A_2‡	6	45.5	82.8 ± 1.9 (6)	100	—
C^2‡	10	45.7	— —	0	11.6 ± 0.6

The values in parentheses indicate the number of animals which survived and on which averages are based.

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

† Experimental period, 28 days.

‡ Each rat in this series received intraperitoneal injections of 200 γ thyroxin 3 times weekly during the course of the experiment.

greater digestion and absorption of the thyroid material. Such, however, does not appear to be the case for subsequent findings indicate the deleterious effects of pancreas feeding may be demonstrated following parenteral administration of thyroxin. The latter experiment was conducted with 16 female rats of the Long-Evans strain similar in age and weight to those employed in the earlier studies. Six of these rats were fed the synthetic ration (died A_2); the remaining 10 were fed a diet containing the activated pancreas preparation (diet C_2). Three times weekly each rat received intraperitoneal injections of 200 γ thyroxin.[‡] Injections were continued for 28 days or until death, whichever occurred sooner. All animals fed the synthetic ration (diet A_2) and receiving the thyroxin injections survived the experimental period of 28 days. All animals fed activated pancreas (diet C_2) and administered thyroxin died (average survival time 11.6 days). These findings indicate that the deleterious effects of pancreas feeding in the thyroid-fed rat were not due to increased digestion or absorption of thyroactive material.

Summary. Immature female rats failed to survive when fed purified rations containing both pancreas and desiccated thyroid. Length of survival was significantly prolonged if either pancreas or thyroid were eliminated from the experimental ration. The deleterious effects of pancreas in the thyroid-fed rat were not due to increased digestion or absorption of thyroactive material.

[‡] Thyroxin (Synthetic Cryst.), Roche-Organon, Inc., Nutley, N. J. The material was dissolved in .1 N NaOH, adjusted to a pH of 8.0 and diluted to a volume containing 200 γ thyroxin per cc.

16638

Production of Hemagglutinin by Mumps and Influenza A Viruses in Suspended Cell Tissue Cultures.[*]

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The property of agglutinating red blood cells characteristic of certain viruses such as mumps and influenza has provided a simple method of estimating their concentration in infected materials. The procedure however, insofar as we are aware, has not been applied to viruses propagated in tissue cultures. Accordingly, experiments were undertaken with the purpose of ascertaining (a) whether the mumps virus would proliferate in suspended cell tissue cultures of the Maitland type and (b) whether this virus and influenza A virus would in such media produce detectable levels of hemagglutinin.

Methods. Preparation and maintenance of cultures. Cultures were prepared in a manner similar to that described by the Maitlands.[1] The nutrient medium consisted routinely of 3 parts of a balanced salt solution made up according to the formula of Hanks[†] and 1 part of Simm's ox-blood serum ultrafiltrate.[‡] This mixture will hereafter be referred to as "HS." In the experiments with mumps virus, the tissue employed consisted of amniotic membrane from 8- or 9-day-old chick embryos. The membranes were suspended in a small amount of "HS", minced with scissors to obtain fragments approximately 1 mm in diameter, and centrifuged for 2 minutes at 800 r.p.m. in a graduated centrifuge tube. The fragments were then resuspended in a total volume of "HS" six times that of the sedimented tissue. Using a large bore Pasteur pipette, 3 drops of this tissue suspension were transferred to 25 cc Erlenmeyer flasks containing 3 cc of "HS" and fitted with rubber stoppers. In the experiments with influenza virus, the cultures were similarly prepared employing amniotic membrane, allantoic membrane, or embryonic brain tissue obtained from 8-day-old eggs. All cultures were incubated at 35°C.

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[*] Aided in part by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Maitland, H. B., and Maitland, M. C., *Lancet*, 1928, **2**, 596.

[†] The balanced salt solution as recommended by Dr. J. H. Hanks, contained the following ingredients expressed as g/l: NaCl, 8.0; KCl, 0.4; $MgSO_4 \cdot 7\ H_2O$, 0.1; $MgCl_2 \cdot 6\ H_2O$, 0.1; $CaCl_2$, 0.14; Na_2HPO_4, 0.06; KH_2PO_4, 0.06; glucose, 1.0. To each liter, 5 cc 0.4% phenol red was added. After autoclaving at 10 lb. for 10 minutes, 0.5 cc 1.4% isotonic $NaHCO_3$ was added per 20 cc of the above.

[‡] Purchased from Microbiological Associates Laboratories, Flemington, N. J.

Their subsequent handling differed from that usually employed with suspended cell cultures in that the nutrient fluid was replaced at intervals as indicated below. This was accomplished by tilting the flasks, allowing the tissue to settle, and removing as completely as possible the supernatant fluid from each flask with a fine bore Pasteur pipette; 3 cc of fresh "HS" were then added. In certain of the experiments subcultures were carried out by transferring aliquots of the pooled supernatant fluids thus removed to flasks containing freshly prepared tissue fragments.

Determination of hemagglutinin and egg infectivity titers. Using a modified Salk technic[2] the hemagglutinin titers were determined of the stock viruses and of the individual supernatant fluids removed periodically from the tissue cultures. The tests on the supernatant fluids were performed as soon as they were removed, using two-fold dilutions and beginning with a final dilution of 1:2. Pooled erythrocytes obtained from the same 4 hens were employed throughout. The highest final dilution of the supernatant fluid giving complete agglutination was taken as the endpoint. The results were expressed as a geometric mean of the individual hemagglutinin titers of the supernatant fluids from each set of cultures.

In the experiments on the growth of mumps virus in tissue cultures, the infectivity for chick embryos of the original inoculum and certain of the supernatant fluids removed at various times was determined. Aliquots of the supernatant fluids were pooled and centrifuged for 15 minutes at 1000 r.p.m. and the sediment discarded. Serial ten-fold dilutions were then prepared in isotonic phosphate buffer (pH 7.1) and 7-day-old eggs in groups of 5 or 6 were inoculated intraamniotically with 0.1 cc of each dilution. Six or 7 days later, the amniotic fluids were harvested and tested individually in a final dilution of 1:8 for the presence of hemagglutinins. The presence of complete hemagglutination in this dilution was taken as evidence of infection. From the data so obtained the 50% infectivity end point was calculated by the method of Reed and Muench.[3] No assay of the infectivity of the cultures inoculated with influenza virus was made.

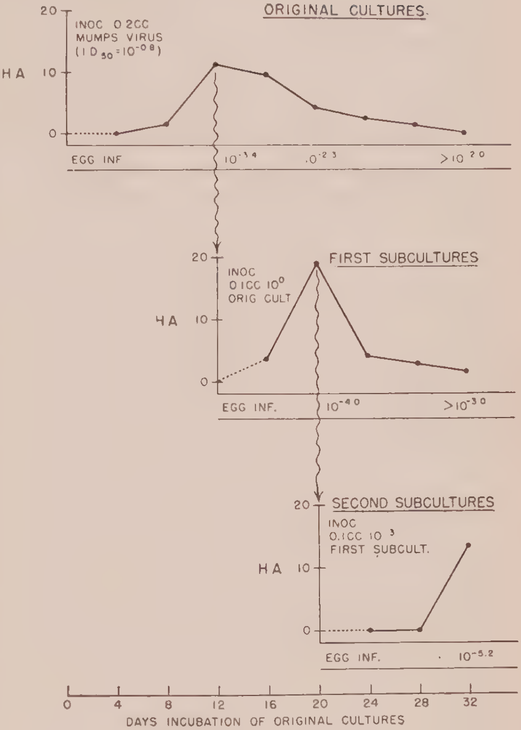

FIG. 1.

Mean hemagglutinin and infectivity titers of tissue cultures inoculated with mumps virus.

The hemagglutinin titer (HA) is expressed as the reciprocal of the geometric mean of the individual titers of the supernatant fluids from 4 cultures. The infectivity titers are expressed as the dilution of the pooled supernatant fluids from 4 flasks representing the ID_{50} dose for embryonated hen's eggs calculated according to the method of Reed and Muench.[3]

Experimental. (1). *Cultivation of mumps*

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[2] Enders, J. F., and Levens, J. H., In: Diagnostic Procedures for Virus and Rickettsial Diseases. American Public Health Association, New York, 1948, pp. 139-164.

[3] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

virus. A typical experiment which is summarized in Fig. 1 will be described. The mumps virus employed as the initial inoculum consisted of pooled amniotic fluid derived from the 42nd egg passage of a strain originally isolated and maintained in this laboratory (Enders strain). This material had a hemagglutinin titer of 512 and an ID_{50} of $10^{-3.8}$ in eggs. Six tissue cultures were prepared with minced amniotic membrane. Four flasks were each immediately inoculated with 0.2 cc of a 10^{-3} dilution in isotonic phosphate buffer of the virus. The two uninoculated cultures were kept as controls. At 4-day intervals the supernatant fluids were removed and 3 cc of fresh "HS" were then added. In this way the original cultures were maintained over a 32-day period. The hemagglutinin titers of all the individual supernatant fluids were determined and the egg infectivity titers of the pooled supernatant fluids removed on the 12th, 20th and 32nd day after inoculation were established.

During this period of 32 days two subcultures were carried out. After the original cultures had been incubated 12 days, with two changes of "HS" in the interim, equal volumes of the supernatant fluids removed on the 12th day were pooled, centrifuged for 15 minutes at 1000 r.p.m. and the sediment discarded; 0.1 cc of the undiluted pooled supernatant fluid was then added to each of 4 newly prepared flasks containing minced amniotic membrane and "HS". The supernatant fluids from the control flasks were handled in an identical manner and 2 new control cultures set up. Flasks of the first subculture group were maintained 20 days with changes of the supernatant fluids at 4-day intervals.

The second subcultures were inoculated with material removed from the first subcultures on the eighth day of incubation. The supernatant fluids from the latter were pooled and centrifuged, diluted 10^{-3} in isotonic phosphate buffer and added in a volume of 0.1 cc to each flask containing newly prepared tissue. The second subculture flasks were incubated for 12 days, with change of the supernatant fluids every 4 days.

(2) *Cultivation of Influenza A virus.* Cultures prepared with minced amniotic membrane, chorioallantoic membrane or brain were inoculated with 0.1 cc of infected allantoic fluid containing the PR8 strain of Influenza A virus diluted 10^{-2} in isotonic phosphate buffer. In one experiment, flasks of each type of tissue were inoculated immediately after they were prepared, while to others the virus was added only after an interval of 6 days. In another experiment the virus was introduced 4 days after the cultures had been prepared and the supernatant fluid changed once. At various intervals, usually each day, 0.5 cc of the supernatant fluid was removed and replaced with the same volume of fresh "HS." The concentration of hemagglutinin in each specimen of fluid was determined.

Results. 1. Mumps virus. The results of the hemagglutinin and infectivity titrations carried out on the supernatant fluids removed from the tissue cultures are summarized in Fig. 1.

From these data it is evident that the mumps virus rapidly increases in cultures of this type. The original cultures were inoculated with 0.2 cc of diluted stock virus having a calculated ID_{50} titer for egg embryos of $10^{-0.8}$; thirty-two days later the ID_{50} titer of the pooled supernatant from the second subculture was $10^{-5.2}$. Not taking into account the quantity of virus discarded in the supernatants and assuming that each routine addition of nutrient fluid resulted in a 1:15 dilution of the virus, it may be calculated from the data included in Fig. 1 that an increase of the order of 1.3×10^{17} occurred in the egg infectivity titer. Likewise, during the experimental period an increase in the hemagglutinin titer of the order of 3.3×10^{14} occurred.

Active tissue growth of apparently the same degree was observed in all flasks irrespective of whether or not the virus had been inoculated. About the 6th to 8th day of cultivation free-floating, thin-walled cyst-like structures became numerous in the cultures of amniotic membrane. The cyst-like forms were no longer apparent after the 20th day. Between the 12th and 16th day, fixation of some of the tissue fragments to the bottom of the flask was noted. These could be seen under low magnification to be surrounded by a zone of proliferating cells. In the cultures maintained 32 days there was an apparent decrease in the rate of growth after the 28th day; at this time many of the fragments previously adherent to the glass became loose and floated in the fluid.

Morphologic evidence of growth was accompanied by a fall in pH. Thus, during the 4-day interval between the changes of nutrient fluid, the pH, as estimated colorimetrically, fell from 7.6-7.7 to 6.9-7.1. This shift occurred throughout each 4-day period until the 28th day of cultivation in the original series. Thereafter, coincident with the apparent decrease in the rate of growth, the pH did not go below 7.3-7.5.

2. *Influenza virus.* The results with 3 different series of cultures have shown that the PR8 strain of Influenza A virus multiplies rapidly in the type of tissue culture employed as indicated by the early appearance and subsequent increase of hemagglutinin in the supernatant fluid. Thus in one experiment in which freshly prepared cultures of allantoic membrane, amniotic membrane and brain were employed, the geometric mean hemagglutinin titers after 72 hours of 2 cultures of each kind of tissue were 91, 6, and 32 respectively. Since the hemagglutinin titer of the virus inoculum was 512 and the dilution factor 3000, it can be calculated that the concentration of hemagglutinin during the period of cultivation had increased 535 times in the cultures of allantoic membrane, and 188 X and 35 X respectively in those of brain and amniotic membrane. The comparatively low titer obtained with the cultures of amniotic membrane should not be regarded as evidence for the failure of the virus to multiply as actively in this tissue, since in other experiments the amount of hemagglutinin produced was equivalent to that encountered in cultures of allantoic membrane and brain. In this instance the fact that relatively small amounts of amniotic tissue were employed probably was responsible for the low yield of hemagglutinin. It is of interest to observe that the embryonic brain tissue supported the multiplication of the virus almost as well as the allantoic membrane. Whereas neurotropic strains of influenza virus have been developed by Stuart-Harris and others[4-7] it is generally held that the PR8 strain has no affinity for nervous tissue.

Similar results were obtained in respect to the increase in hemagglutinin in tissue cultures which had been maintained for 4 or 6 days before the virus was inoculated. On the whole, however, the titers were somewhat lower. For example, 72 hours after inoculation of the virus, the geometric mean titers of two cultures of each of the 3 kinds of tissue were 8, 11, and 6 for allantoic membrane, amniotic membrane and brain respectively. These cultures had been prepared 6 days before the virus was added. Similarly in cultures of allantoic membrane prepared 4 days before inoculation the mean hemagglutinin titer did not exceed 16 after 72 hours.

Determinations of hemagglutinin titers at 12, 16, 20, 24, 48 and 72 hours and at certain intervals thereafter showed that the maximum production was attained between the 2nd and 3rd day. The hemagglutinin was present, however, in low concentration as early as 16 hours. By removing on the 4th day following inoculation all the supernatant fluid and replenishing with fresh material, it was found that thereafter a slow increase in hemagglutinin may gradually occur. But the titer attained did not exceed 2 even after an interval of 10 days and this was observed only in a series of cultures that were inoculated with the virus immediately after their preparation.

Discussion. It has been demonstrated that an egg-adapted strain of mumps virus multiplies actively in tissue cultures consisting of fragments of amniotic membrane of the chick embryo suspended in a mixture of ox serum ultrafiltrate and balanced salt solution. In

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[4] Stuart-Harris, C. H., *Lancet*, 1939, **1**, 497.

[5] Francis, T., Jr., and Moore, A. E., *J. Exp. Med.*, 1940, **72**, 717.

[6] Henle, G., and Henle, W., *Science*, 1944, **100**, 410.

[7] Vilches, A., and Hirst, G. K., *J. Immun.*, 1947, **57**, 125.

this medium its multiplication is sufficient to produce measurable quantities of hemagglutinin in the supernatant fluid even when small inocula are employed. It is therefore possible within certain limits to estimate with ease the capacity of the virus to multiply under these conditions. It has also been determined that Influenza A virus (PR8 strain) increases in this type of medium to a level at which its hemagglutinating property can also be readily assayed.

The development of measurable amounts of hemagglutinin in tissue cultures is of significance, since it provides a swift and convenient means of following the rate and degree of multiplication of the mumps and influenza viruses in a system which, compared with a medium such as the chick embryo or the mouse, is far less complex. The technic, therefore, might be applied profitably to the study of many problems, for example, the effect of various chemical compounds in accelerating or inhibiting the growth of these viruses.

It is noteworthy that the rate of multiplication of influenza A virus was found to be much more rapid than that of mumps. This difference has been previously observed in experiments with infected chick embryos and would seem, as Ginsberg, Goebel and Horsfall[8] have indicated, to reflect a basic difference in the biologic properties of the two agents.

Conclusions. 1. An egg-adapted strain of mumps virus has been cultivated in suspended cell tissue cultures. 2. In such media the mumps virus as well as Influenza A virus (PR8 strain) produce measurable quantities of hemagglutinin.

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[8] Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., *J. Exp. Med.*, 1948, **87**, 385.

16639

Magnesium Deficiency in the Sexually Mature Rat.*

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That the young rat requires an adequate dietary intake of magnesium for normal life processes was first established by Kruse, Orent, and McCollum.[1] As shown by Tufts and Greenberg, two phases seem to be observed in magnesium deficiency in young rats.[2] The first is marked by vasodilatation, hyperemia, and hyperexcitability. The second phase is characterized by nutritive failure, cachexia, and kidney damage. There have been controversial reports as to the pathological involvement of the skin in magnesium deficiency in rats. Kruse and his co-workers reported definite trophic changes in the skin and nails of young animals on a diet deficient in magnesium. Likewise, other workers reported skin lesions in animals deprived of magnesium.[3,4] On the contrary, Tufts and Greenberg and others did not observe disturbances in the skin of young animals raised on diets deficient in magnesium.[5,6] Greenberg suggested that the trophic conditions observed by several workers were due to the experimental animals not receiving various constituents of the vitamin B complex in sufficient quantities.[7]

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* Aided by a grant from the Dow Chemical Company, Freeport, Texas, through the Texas A & M Research Foundation.

[1] Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1932, **96**, 519.

[2] Tufts, E. V., and Greenberg, D. M., *J. Biol. Chem.*, 1938, **122**, 693.

[3] Watchorn, E., and McCance, R. A., *Biochem. J.*, 1937, **31**, 1379.

[4] Shrader, G. A., Prickett, C. O., and Salmon, W. D., *J. Nutrition*, 1937, **14**, 85.

[5] Brookfield, R. W., *Brit. Med. J.*, 1934, **1**, 848.

TABLE I.
Effect of Magnesium on the Weights of Sexually Mature Rats.

Dietary treatment Mg	No. of animals	Initial wt g	Final wt g	Gain per wk g
+	3	156	337*	13.9
—	3	147	249	7.7
+	3	311	385†	14.2
—	8	311	346	7.0
+	4	215	283†	13.6
—	7	209	245	7.0

* Weight after a period of 8 weeks.
† Weight after a period of 5 weeks.

Though the manifestations of magnesium deficiency in the young rat have been well established, the specific effect of magnesium deprivation in the older rat is still questionable. The experiments reported here were designed to determine the effect of insufficient dietary magnesium on sexually mature rats.

Experimental. The composition of the diets used has been reported in a previous paper.[8] The magnesium low diet contained less than 1.0 mg of magnesium per 100 g, while the control diets contained approximately 30 mg per 100 g. Three different series of rats were used with average beginning weights of 152, 311, and 212 g respectively.

Vasodilatation and hyperemia were not outstanding symptoms in the sexually mature rats on the magnesium-deficient diet. In several animals none was observed at all, but in most instances these symptoms made their appearances 14 to 19 days following the start of the experiment. The vividness of the red color of the ears and extremities during this period was not pronounced, but the slight reddening of these areas was easily detected and usually was observed for 5 to 6 days with a gradual fading towards the end of this period. This increased length of time before vasodilatation and hyperemia could be observed and the lack of marked vasodilatation are differences between the young animals and the sexually mature animals. It has been shown that young rats on a magnesium-deficient diet show vivid reddening of the ears and extremities within 3 to 5 days after being restricted to the deficient diet.

The animals on the magnesium-deficient diet showed only a slight degree of nervousness and apprehensiveness during the period of vasodilatation and hyperemia, but increased nervousness was noticed 4 to 7 days after vasodilatation had subsided. No cases of spontaneous convulsions were observed.

Significant differences in the average weight gains of the experimental and control animals were noted as early as the second week. By the end of the 5th week the control animals had an average gain of almost twice that of the experimental animals. After 8 weeks on the regimens for the first series and 5 weeks for the second and third series some of the animals on the deficient diet were transferred to the magnesium-supplemented diet. These animals showed an immediate response in rate of gain in weight while those on the deficient diet made only slight gains or lost weight.

After approximately 3 weeks on the experimental diets, edema was observed in the nasal regions of the animals on the deficient diet. The hair became sticky, stiff, and lusterless. Occasionally a sticky, reddish exudate was seen on the feet, noses and ears. Erythematous changes in the skin began to appear between the 23rd and 28th day after the experiment had started. The erythematous changes were characterized by small circular areas on the dorsum of the back or neck in

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[6] Cramer, W., *Lancet*, 1932, **223**, 174.

[7] Greenberg, D. M., *Ann. Rev. Biochem.*, 1939, **8**, 269.

[8] Kunkel, H. O., and Pearson, P. B., *Arch. Biochem.*, 1948, **18**, 461.

most of the animals. This skin condition appeared to become progressively worse with time, and erythema was noticed around the scrotal area in the males, on the abdomen, and on the sides and under the jaws of animals of both sexes. Following the erythema, purpural skin hemorrhages were observed in the areas which had shown erythema. Extensive eschar formation followed the purpura. Usually the eschars were hard and dry, and the escharotic tissue was confined to small circumscribed areas. Exfoliation followed the escharotic stage, and there was a loss of hair around the areas previously occupied by the eschars. In some cases, ulceration followed exfoliation. When this occurred eschars were usually reformed, and exfoliation generally followed.

Those animals that were transferred to the control diet showed rapid improvement and recovery from the skin lesions. In cases where eschars were present, exfoliation and healing took place rapidly. Denuded areas began to show hair growth in 7 to 10 days, and these areas could not be detected at the close of the experimental period without close examination which would reveal numerous short hairs. Edema subsided within a week after the animals received the increased dietary magnesium, and the animals appeared in good condition by the close of the experiment.

Summary. Sexually mature rats maintained on a magnesium-deficient diet develop lesions of the skin characterized by erythema, purpural hemorrhages and eschars. The addition of magnesium to the diet results in recovery from the skin lesions. Other symptoms observed are essentially the same as in the immature animal deprived of magnesium. The increased length of time before the appearance of symptoms in older animals probably reflects increased body stores of magnesium with an increment in the time required to deplete the tissues of this element.

16640

Effect of Aminophyllin on the Coagulation of Human Blood.*

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Aminophyllin (theophyllin-ethylenediamine) is commonly used in the treatment of patients with heart disease, particularly when the coronary arteries are involved. Recently, emphasis has been put on the importance of intravascular coagulation of the blood on the course and prognosis following coronary occlusion. Observations have been published ascribing to aminophyllin a thromboplastic action. Because of the serious therapeutic implications of this finding, if confirmed, the present study was undertaken. Field, Larson, Spero, and Link[1] in reviewing the literature, found that a number of authors were of the opinion that the methylxanthines had an accelerating action on blood coagulation. They also reported that single oral doses of a number of methylxanthines produced hyperprothrombinemia and a decreased sensitivity to the action of 3,3′-methylenebis-4-hydroxycoumarin. In view of these findings they believed it possible that use of these drugs in man might produce a tendency toward thrombus formation. Link[2] again emphasized

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We are indebted to Dr. John W. Fertig, Professor of Biostatistics, Columbia University College of Physicians and Surgeons, for making the statistical analyses.

[1] Field, J. B., Larsen, E. G., Spero, L., and Link, K. P., *J. Biol. Chem.*, 1944, **156**, 725.

[2] Link, K. P., *Harvey Lectures*, 1943-44, **39**, 126.

these points and suggested that the use of the dilute (12.5%) plasma prothrombin time determination was preferred in attempting to evaluate any such changes in man. On the other hand, Poindexter and Meyers[3] found that oral administration of aminophyllin to a series of 10 patients had no effect on the plasma prothrombin time. Breytspraak and Greenspan,[4] in a small series of cases, found that oral and intravenous administration of aminophyllin produced no significant changes in the prothrombin time. Gilbert, Dey, and Trump[5] performed a series of experiments on both patients and animals and found no alteration in the clotting times, dilute and 100% plasma prothrombin times, or in the response to intravenous injection of heparin following the administration of xanthine drugs.

Material. A single intravenous injection of aminophyllin was given to 18 patients on the wards of the hospital. None had any liver disease or disorder of the blood-forming organs and in no case were drugs being taken which are known to affect the clotting of the blood. Following a control determination of clotting and prothrombin times, each subject was given 0.48 g of aminophyllin by intravenous injection over a 10-minute period. Clotting and prothrombin times were determined 30 minutes and one hour after completing the injection. Moderate tachycardia and diuresis were noted in most cases, but there were no other effects.

It is possible that aminophyllin has a slower action on blood clotting than would be detected following a single intravenous injection. Accordingly, a second group of 12 patients was studied to observe a more prolonged action of aminophyllin on blood coagulation. As in the first group, none of these patients had disease affecting the clotting of the blood nor were any taking drugs with known thromboplastic action.

Following an initial determination of clotting and prothrombin times, each patient was given 0.2 g of aminophyllin, by mouth, 4 times a day for 7 days. Two patients were given plain aminophyllin tablets, but because of nausea the remaining 10 patients took an enteric-coated preparation. At the end of the period of administration, a second determination of clotting and prothrombin times was done. With the enteric-coated preparations no ill effects were noted. Three patients, who had angina pectoris due to coronary insufficiency, obtained marked symptomatic relief while taking the aminophyllin.

Methods. These are described at length because strict attention to technical detail is essential to obtain accurate and consistent results. The clotting time was determined by a modification of the Lee-White method.[6] The equipment consisted of three 75 × 10 mm tubes in a water bath maintained at a constant temperature of 36 to 39°C. These tubes were cleaned chemically, rinsed in distilled water, and oven-dried before being used. Venous blood was obtained with as little trauma as possible and was collected in a dry syringe. The tourniquet was released before withdrawal of the blood. The stopwatch was started when one-half the required amount of blood had been withdrawn. One cc of this venous blood was placed into each of the 3 tubes. At 3 minutes the first tube was tilted and this procedure was repeated every 30 seconds until the tube could be tilted to the horizontal without having the blood flow down the side. At that time the second tube was similarly tilted and finally the third tube. The end point was considered to be that point at which the third tube could be tilted to the horizontal without any flow of blood.

The prothrombin time was determined by a slight alteration in the Link-Shapiro modification of Quick's method.[7] Five cc of venous blood was collected into a chemically clean test tube containing 0.01 g of solid potassium oxalate. After mixing, this sample was centrifuged and the plasma pipetted off and placed in a 75 × 10 mm test tube. Fifty

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[3] Poindexter, C. A., and Meyers, L., *Quarterly Bull. Northwestern Univ. Med. School,* 1946, **20**, 130.

[4] Breytspraak, R. W., and Greenspan, F. S., *Am. J. Med. Sci.,* 1946, **212**, 476.

[5] Gilbert, N. C., Dey, F. L., and Trump, R. A., *J. Lab. and Clin. Med.,* 1947, **32**, 28.

[6] Lee, R. I., and White, P. D., *Am. J. Med. Sci.,* 1913, **145**, 495.

[7] Shapiro, S., *Exp. Med. and Surg.,* 1944, **2**, 103.

TABLE I. Clotting and Prothrombin Times Before and After Administration of 0.48 g of Aminophyllin by Intravenous Injection.

Diagnosis	Lee-White clotting time of blood (minutes)				Undiluted plasma prothrombin time (% of normal control)				12.5% plasma prothrombin time (% of normal control)			
	Initial	½ hr	1 hr	Final change	Initial	½ hr	1 hr	Final change	Initial	½ hr	1 hr	Final change
Acute rheumatic fever	7.5	8.5	7.0	— 0.5	100.0	123.5	110.5	+10.5	95.5	95.8	96.6	+ 1.1
Pneumonia	10.0	8.0	9.0	— 1.0	124.1	112.7	106.0	—18.0	84.1	92.3	89.6	+ 5.5
Secondary syphilis	6.5	7.5	7.5	+ 1.0	95.2	112.9	116.3	+21.1	100.2	98.2	107.5	+ 7.3
Diaphragmatic pleurisy	8.0	8.0	10.0	+ 2.0	116.4	107.6	108.7	— 7.7	81.1	79.1	80.1	— 1.0
Lymphadenitis	7.0	7.5	6.5	— 0.5	92.9	98.1	91.1	— 1.8	109.1	116.7	115.3	+ 6.2
Pneumonia	8.0	9.5	10.5	+ 2.5	107.6	100.5	105.9	— 1.7	93.1	98.0	85.1	— 8.0
Coronary insufficiency	9.0	8.5	9.5	+ 0.5	109.0	98.4	96.4	—12.6	104.1	93.4	90.6	— 3.5
Trichinosis	6.5	7.5	6.5	± 0.0	104.0	98.2	101.3	— 2.7	103.9	101.9	104.8	+ 0.9
Pneumonia	6.5	7.5	6.5	± 0.0	105.6	101.2	104.3	— 1.3	98.7	101.4	102.1	+ 3.4
Lymphoblastoma	5.5	5.5	5.0	— 0.5	86.1	91.3	95.1	+ 9.0	91.0	97.3	99.2	+ 1.2
Pneumonia	6.5	6.5	7.0	+ 0.5	105.1	111.8	112.7	+ 7.6	97.3	104.9	106.4	+ 9.1
Psychoneurosis	5.0	4.5	4.5	— 0.5	92.3	96.1	91.7	— 0.6	87.7	92.4	93.9	+ 6.2
Pneumonia	7.0	7.0	7.5	+ 0.5	114.8	114.8	124.2	+ 9.4	111.5	100.7	101.4	—10.1
Coronary heart disease	7.0	6.5	6.0	— 1.0	98.8	96.1	106.6	+ 7.6	93.3	100.8	98.6	+ 5.3
Pneumonia	9.5	9.5	10.0	+ 0.5	94.2	95.2	104.7	+10.5	87.4	85.7	85.2	— 2.2
Nasal polyposis	9.0	9.5	9.0	± 0.0	99.4	96.0	102.8	+ 3.4	98.8	100.8	89.7	— 9.1
Amebic dysentery	8.5	9.0	8.5	± 0.0	92.7	98.6	89.0	— 3.7	111.6	105.3	104.2	— 7.4
Duodenal ulcer	7.0	7.5	8.0	+ 1.0	93.6	96.4	97.1	+ 3.5	94.7	88.8	90.0	— 4.7

TABLE II.

Clotting and Prothrombin Times Before and After Administration, by Mouth, of 0.2 g of Aminophyllin, Four Times Daily for One Week.

Diagnosis	Lee-White clotting time of blood (min)			Undiluted plasma prothrombin time (% of normal control)			12.5% plasma prothrombin time (% of normal control)		
	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change
Coronary heart disease	7.5	7.5	± 0.0	102.7	87.0	—15.7	106.2	105.1	— 1.1
" " "	5.5	9.5	+ 4.0	97.2	111.2	+14.4	94.1	99.4	+ 5.3
" " "	5.0	5.5	+ 0.5	107.1	107.2	+ 0.1	91.4	94.2	+ 2.8
" " "	8.5	8.5	± 0.0	109.8	98.2	—11.6	103.4	96.3	— 7.1
Hypertensive vascular disease	6.0	4.5	— 1.5	91.0	84.3	— 6.7	96.7	91.8	— 4.9
Cor pulmonale; pulmonary fibrosis	7.0	8.0	+ 1.0	78.7	97.1	+18.4	84.5	88.7	+ 4.2
Tuberculous pericarditis	5.5	5.5	± 0.0	75.4	100.5	+25.1	91.4	96.2	+ 4.8
Coronary heart disease	4.5	8.0	+ 3.5	88.4	87.5	— 0.9	88.4	90.0	+ 1.6
Thoracic neoplasm	6.0	8.0	+ 2.0	87.9	96.7	+ 8.8	93.1	99.3	+ 6.2
Hypertensive cardio-vascular disease	6.0	8.0	+ 2.0	113.6	109.8	— 3.8	107.0	118.3	+11.3
Regional enteritis	5.5	7.0	+ 1.5	98.9	113.1	+14.2	116.7	101.7	—15.0
Coronary heart disease	4.5	6.5	+ 2.0	101.1	105.8	+ 4.7	84.4	96.0	+11.6

mg of desiccated rabbit lung[†] was placed into 2.5 cc of normal saline solution and stirred for 10 minutes in a water bath at 56 to 58°C. The mixture was then cooled at 26°C for 1 minute. Two and one-half cc of 0.025 molar calcium chloride were added to this thromboplastin suspension and the resulting mixture stirred for an additional 4 minutes. The thromboplastin-calcium chloride mixture was then centrifuged for 5 minutes, the supernatant decanted and placed in 0.2 cc amounts into 75 × 10 mm test tubes.

The actual test was performed by adding 0.1 cc of the plasma to the 0.2 cc of thromboplastin-calcium chloride mixture and stirring the solution vigorously with a wire loop. The stop-watch was started as the first drop of plasma entered the thromboplastin tube. The end point was that time at which a firm clot formed across the wire loop. An identical test was also done using 12.5% plasma. This was obtained by diluting a sample of the plasma 1 to 8 with normal saline solution.

The strength of the thromboplastin was found to vary considerably with different lots of the preparation and for this reason a control plasma prothrombin time was determined on a normal subject at the time each patient was tested. In order to orient the results in relation to a common baseline, the prothrombin times are all expressed as the percentage of the normal control for a given day. Duplicate determinations were made on both undiluted and 12.5% plasma, in the control as well as in the subject whose blood was being studied.

Results. In the group given a single dose of 0.48 g of aminophyllin by intravenous injection there was no significant change in the clotting times. The individual and average variations were slight. The average initial clotting time was 7.5 minutes. After one-half hour this had increased to 7.7 minutes; the final average was the same, indicating an increase in the clotting time of 0.2 minute (Table I). In the patients on prolonged oral aminophyllin intake there was also very little individual or average variation. The average initial clotting time was 6.0 minutes and the final 7.2 minutes, indicating an increase of 1.2 minutes (Table II).

In the patients who had received aminophyllin by intravenous injection there was little difference in the prothrombin times before and after administration of the drug. The average initial undiluted plasma prothrombin time was 101.8%, at one-half hour it was 102.8%, with a final average of 103.5%, indicating an overall increase of 1.7%. Similar results were obtained using 12.5% plasma. In this case the initial average was 96.8%, with a one-half hour average of 97.4% and a final average of 97.2%. This was a final increase of 0.4%. In the group of patients receiving aminophyllin orally for one week there was again little variation in the prothrombin times before and after drug administration. The initial undiluted plasma determination averaged 96.0% with a final average of 99.9%, indicating an increase of 3.9%. Similarly, in the determination on 12.5% plasma the initial average was 96.4% and the final 98.1%; this was an increase of 1.7%. In none of the results were the differences statistically significant.

Summary. Aminophyllin was given in a single intravenous injection to a group of 18 patients, and orally 4 times daily for a week to a second group of 12 subjects.

There were no statistically significant changes in the clotting time of the blood or in the undiluted or 12.5% (diluted) plasma prothrombin times following the administration of aminophyllin.

[†] Prepared by the Maltine Company, New York.

16641

Preparations of Lysates from Cultures of *T. cruzi* and Their Effects on Normal and Tumor-Bearing Mice.

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(Introduced by A. F. Coca.)
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Klyueva and Roskin[1,2] have reported the production of an extract of *T. cruzi* which had a specific cancerolytic effect on experimental tumors in mice. Attempts to confirm these findings were unsuccessful.[3,4,5] The present report is a summary of experiments done in an attempt to verify the findings of the Soviet investigators employing a whole culture lysate.

Materials and Methods. *T. cruzi* cultivation and preparation of *T. cruzi* extracts: Cultures* were grown on NNN agar with an overlay of Tyrode's solution and incubated at 24°-25°C. At the height of growth, the concentration of organisms was determined as follows: A 4 mm loopful of culture was examined at 250× magnification. Fifty fields were examined and the average number of organisms per field divided by two represented the concentration of organisms in millions per ml. (Repeated comparisons with hemocytometer counts confirmed the reliability of this method.) Dilutions were based on units, one unit representing one million organisms.

The cultures were diluted with distilled water and metaphen (Abbot) so that each ml contained 4 units of *T. cruzi* lysate and metaphen in a concentration of 1:10,000. After storage at 8°C for 24 hours, the trypanosomes were found to have undergone lysis. This was confirmed by microscopic study of the diluted culture which was also examined for sterility. The culture lysates were stored at –20°C until used, except for one experiment in which they were kept at 8°C. None were kept more than 6 days. If not used in this period, the lysate was discarded.

Treatment. These lysates were injected into mice of varying strains and bearing several types of tumors. The injections were always given subcutaneously at a site as far from the tumor as possible. The mice were housed in individual jars and fed a prepared complete pellet diet.

The control animals received injections of sterile medium diluted with the same amount of distilled water and metaphen as the lysates. The number of injections and amounts of solution injected were the same for the control and treated groups.

The following observations were recorded:

(a) Body weight twice weekly.

(b) Tumor size 3 times weekly. (Two diameters were measured by calipers and a drawing representing the area of the tumor was made on the individual records maintained for each mouse.)

(c) Any change in the tumor or in the general condition of the animal was noted.

(d) At necropsy when the animal died or was sacrificed, gross and microscopic examinations of the tumor, liver, kidney, intestine, and heart were made. (Zenker's fixation—H. and E. stain.)

(e) Stool culture studies were made on the Bagg-albino strain of mice used to control subclinical enteric infections. No infections were found throughout the entire series.

Findings. To determine the presence of any toxic factors, injections of the *T. cruzi*

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[1] Shimkin, Michael B., Surgeon, U. S. Public Health Service, personal communication, 1947.

[2] Klyueva, N. G., *Am. Rev. Soviet Med.*, IV, June, 1947, p. 408.

[3] Hauschka, T., Saxe, L. H., Jr., and Blair, M., *J. Nat. Cancer Inst.*, 1947, **7**, No. 4.

[4] Hauschka, T., and Goodwin, M. Blair, *Science*, 1948, **107**, 600.

[5] Cohen, A. L., Borsook, H., and Dubnoff, J. W., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 40.

* Obtained from Dr. M. H. Soule, University of Michigan, and designated by us and by the National Inst. of Health, Tropical Disease Division, as the "Soule" or "S" strain.

TABLE I.

Treatment of Spontaneous Mammary Cancer Mice with *T. cruzi* Lysate.[1] Animal Strain the Bagg Albino.[2]

Exp.	Tumor size at start test	Dosage: No. of inject. (subcut.)	Total tumors		% No effect		% inhibition		% Regression		% Deaths	
			Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
A	1 cm or less	45 (total 45 u.)	107	86	69	88	19	8	12	4	50	30
B	>1 cm	45 (total 45 u.)	51	51	86	88	12	10	2	2	71	52
C	1 cm or less	5[3] (total 15 u.)	37	33	24	54	52	27	24	19	S[4]	

[1] Controls treated with equivalent volume and dilution of sterile medium.
[2] Obtained from Mrs. F. O'Grady, Bronx, N. Y. C.
[3] Sacrificed 48 hours after 5th injection.
[4] Sacrificed.

lysate were given to normal mice and a variety of tumor-bearing mice. Only occasional evidence of liver and kidney damage was noted despite the fact that the average survival time of cancerous mice treated with *T. cruzi* lysate was less than the controls injected with sterile medium.

No gross or microscopic differences in the treated tumor-bearing animals of the A strain mice (spontaneous mammary carcinoma[†] and transplanted carcinoma No. 119[‡]) were observed as compared with the same types of tumor-bearing mice treated with sterile medium.

This was also true of the spontaneous mammary carcinoma larger than one cm in size of the Bagg-albino strain. (Table I, Experiment B.) However, in this latter strain where the same tumor was less than one cm in size there was a greater degree of inhibition in the treated groups as compared with the control groups. The per cent of these mice showing no effect was: controls 71%, treated 46%. (Combined findings of Experiments A and C, Table I.) This finding was not considered sufficiently significant to state that the lysate of *T. cruzi* exerted a specific cancerolytic effect. However, the association of this finding with the fact that the survival time of the treated tumor-bearing animals was distinctly less than that of the control tumor-bearing animals does indicate that the lysate may contain some active principle that exerts an effect on tumor-bearing animals, and that this effect was observed only in mice with tumors less than one cm in size. Whether or not this effect is a direct or indirect one is beyond the scope of this study. The differences observed could not be attributed to any nutritional effect since 80% of the animals surviving the experiment showed no weight loss. Those which lost weight were equally divided between treated and control groups, and tumor sizes of all of these latter mice were approximately the same.

In another group of Bagg-albino mice with spontaneous mammary carcinoma one cm or less, 5 daily injections were given, the dose being increased by one unit each day. Forty-eight hours after the last injection, each animal having received a total dose of 15 units, the mice were sacrificed. The results were considered comparable to those obtained in similar mice receiving 45 daily injections (See Table I).

A large series of experiments were performed using mice of the Bagg-albino strain with implanted sarcoma No. 180. This tumor proved to be completely unreliable, both controls and treated animals showing a high percentage of complete regressions. Of a group of 27 mice which received no injections, 19 showed complete disappearance of the implanted tumor after the tumor had reached a size of one cm in largest diameter.

[†] Obtained from Dr. M. J. Shear, National Cancer Institute, Bethesda, Md.

[‡] Obtained from Dr. T. S. Hauschka, Lankenau Hospital, Philadelphia, Pa.

These results do not confirm those of Klyueva and Roskin as given in the fragmentary reports available to us. At no time did we have detailed information regarding their methods of *T. cruzi* cultivation and lysate preparation. It is possible that the discrepancy may be due to differences in the ultimate nature of the lysate.

Summary. 1. No inhibition of tumors was noted in Bagg-albino strain mice with spontaneous mammary carcinoma over one cm in size when treated with whole culture lysate of *T. cruzi;* on the other hand in the same mouse strain with tumors under one cm in size a degree of inhibition was noted which warrants closer study.

2. The inhibition noted could not be attributed to malnutrition effects or concurrent bacterial infections.

3. No inhibition was noted in tumor-bearing "A" strain mice (spontaneous mammary carcinoma; transplanted carcinoma No. 119) when treated with *T. cruzi* whole culture lysates.

4. Survival time of treated tumor-bearing mice was less than that of control treated animals.

5. A simple method is given for a reproducible preparation of *T. cruzi* lysates.

The authors gratefully acknowledge the assistance of Alvin Haber, histologist; Leona Caroline, microbiologist; Doris Koopman, Estelle Goldman and Ann Voltman, student assistants from Antioch College.

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Propagation of Rabbit Fibroma Virus in the Embryonated Egg.*

MARGARET H. D. SMITH. (Introduced by R. E. Shope.)

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In view of the theoretical interest attached to the relationship of viruses to neoplasms,[1] it seems worth while to report the adaptation to growth in the embryonated egg of a virus which, if it does not cause a true tumor, is at least the etiologic agent of a closely related tumor-like condition. Adaptation of such a virus to a different and relatively simple experimental host such as the developing chick embryo may provide a means of studying the virus-tumor relationship. In fact the earliest use of the chick embryo in the study of viruses was for just this purpose, when Murphy and Rous obtained growth of a fowl sarcoma on inoculating into the chick embryo either minced tumor tissue or a Berkefeld filtrate of such tissue.[2] The virus studied here is that of rabbit fibroma isolated by Shope[3] from a growth on the foot of a wild rabbit. Passed since then in series through domestic rabbits, one strain (the so-called OA strain) has maintained its ability to produce tumors on subcutaneous or intratesticular inoculation. From this original strain there arose a variant (the so-called IA strain described by Andrews[4]) which had lost the ability to produce tumors and caused only inflammation.

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* Part of work reported here was carried out while the author was a National Research Council Fellow in the Medical Sciences.

[1] Rous, P., *Bull. New York Acad. Med.*, 1947, **23**, 65.

[2] Murphy, J. B., and Rous, P., *J. Exp. Med.*, 1912, **15**, 119.

[3] Shope, R. E., *J. Exp. Med.*, 1932, **56**, 793.

[4] Andrewes, C. H., *J. Exp. Med.*, 1936, **63**, 157.

After many unsuccessful attempts at growing strains of the fibroma virus in tissue culture, Faulkner and Andrewes[5] finally succeeded in adapting the IA strain to growth in cultures of rabbit testis.

Paschen,[6] was unable to maintain the IA strain on the chorioallantoic membrane for more than one passage, although the OA strain was still present after 5 passages. However methods and findings are not given in any detail.

Experimental. Methods. The *strain of virus* employed in most of our experiments was the OA, or tumor-producing strain, obtained from Dr. R. E. Shope as glycerinated rabbit testicle. The IA strain was used similarly in the form of glycerinated rabbit testicle. *Titrations* were carried out by grinding a weighed piece of tissue with sand in a mortar and adding sufficient cold veal infusion broth or saline to make a 10% suspension. Further tenfold dilutions were then made in cold broth, and inoculated subcutaneously in a rabbit in 1.0 cc amounts, no more than 9 tests being carried out in any one animal. The rabbits were observed for appearance of subcutaneous tumors at the site of inoculation. *Neutralization* tests were carried out according to Shope's[7] method, *i.e.* a mixture of 1 cc of serum and 1 cc of infection suspension was incubated at 37°C for 1 hour, stored for 2 hours in the refrigerator, then injected in 1 cc amounts into the subcutaneous tissue of a rabbit, together with appropriate controls in the same rabbit.

Results. Adaptation of the Virus to Eggs. The stored glycerinated testicle virus was well washed in cold saline, then ground with sand and saline to make a heavy suspension, which was used to infect a fresh rabbit, 0.5 cc being inoculated into each testicle. Seven days later, when scrotal and testicular edema were pronounced, the animal was killed, small pieces of testicle were ground with sand and sufficient cold saline to make a 1% suspension by weight. After this suspension had been allowed to settle for about an hour in the refrigerator, 0.1 cc of the suspension was dropped onto the chorioallantoic membrane of 4 12-day-old eggs and was also cultured on a blood agar slant. Three days later, after incubation at 35°C, the membranes were harvested, ground lightly in a pyrex glass grinder, together with 4 cc of saline per membrane. After standing for half an hour the supernatant of this suspension was used as inoculum for a new set of eggs. Passages were made in this fashion at regular 3-day intervals. The membrane suspension from the 7th egg passage produced typical fibroma in a rabbit. The egg passage virus having unfortunately become contaminated at the 8th passage, the testicle from the rabbit injected with 7th passage egg membrane suspension was used as a source of virus. The virus was then maintained in eggs for 18 consecutive passages without mishap. In this second series membranes of the 1st, 5th, 10th, 14th, 15th and 18th passages were found to be infectious for rabbits.

Numerous attempts were made to adapt the virus to growth on the chorioallantoic membrane. In each case where virus from fresh tissues was employed it multiplied in the egg. However glycerinated virus did not always establish itself. In some cases the virus passage was discontinued after only 3 or 4 transfers, in others it was carried through 10 or 12 transfers. It was found that the size of the inoculum could be varied between 0.1 and 0.35 cc, the membranes could be ground in physiological saline, saline buffered with phosphate, veal infusion broth, or even egg fluid, though the latter was less satisfactory. It was, however, found necessary to make passages quite regularly at 3-day intervals. The titer of virus was lower on the 2nd and 4th days than on the 3rd, so that passage at 4-day intervals resulted in loss of the virus within 2 to 3 passages. Eggs which had been incubated for 11 days prior to inoculation sustained the growth of the virus most satisfactorily, regularly yielding membranes which were infectious for rabbits when diluted 10^{-4} or 10^{-5} by weight.† The titers obtained with 9-day eggs were slightly lower and the titers with 13-day eggs approximately 100-fold lower.

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[5] Faulkner, G. H., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1935, **16**, 271.

[6] Paschen, E., *Zent. Bakt.*, 1936, **138**, 1.

[7] Shope, R. E., *J. Exp. Med.*, 1932, **56**, 803.

In later passages, whenever possible contamination was feared, penicillin (165 units per egg) and streptomycin (0.0015 g per egg) were added to the membrane suspension.

Viability of Egg Passage Virus. Suspensions of membranes made up in veal infusion broth pH 7.3 to which 20% rabbit serum was added were found to withstand quick freezing with dry ice and alcohol and storage in the dry ice chest for at least 2 months. Suspensions in saline, in egg fluid or even in plain broth were often inactive on thawing. Virus suspensions allowed to stand overnight in the refrigerator showed a roughly 100-fold fall in titer.

Distribution of Virus within the Egg. On several occasions the embryos, yolk sacs, and pooled egg fluids from infected eggs were tested for virus by subcutaneous inoculation into a rabbit. Even when the chorioallantoic membrane suspension proved infectious to rabbits when diluted 10^{-4} or 10^{-5}, no virus was detectable in any other part of the egg.

Routes of Inoculation. Virus which had been through a number of passages on the chorioallantoic membrane was injected in 0.5 cc amounts into the yolk sac. Embryos and yolk sacs harvested 3 to 5 days later contained no demonstrable virus.

Effect of Virus upon the Chick Embryo and its Membranes. In view of the widespread lesions produced on the chorioallantoic membrane by the related myxoma virus,[8,9] it was a disappointment to find no consistent and recognizable effect of fibroma virus on the chick embryo. Many membranes which were shown by rabbit inoculation to contain virus in large amounts appeared as delicate in structure as membranes from normal eggs. On the other hand it seemed, in the course of adapting virus from a number of individual rabbits to growth in the egg, as if the strains derived from some rabbits showed more tendency to cause marked edema of the membrane than did other strains. Passages were made in parallel from edematous and from "non-edematous" membranes. Sometimes the edema-producing capacity would remain for a passage or two before fading out. Once it had disappeared it would not reappear in the course of further passages. The nature of this "lesion" is being subjected to further study at the present time.

Sections of a number of membranes were studied histologically. The spindle shaped cells with large nuclei characteristic of fibroma in the rabbit[3,10,11] could not be demonstrated in the egg. No inclusion bodies were seen. Contrary to the experience reported by Paschen[6] we were unable to demonstrate elementary bodies with certainty on smears stained with Morosow's stain.

The embryos of eggs infected with fibroma virus appeared normal. Several batches of infected eggs were allowed to hatch. The chicks from these eggs were not tested for presence of virus; however, serum obtained from them at the age of 15 days after hatching failed to neutralize rabbit passage fibroma virus. This lack of detectable immunologic response may well be due to the well-known fact that immature animals respond poorly to antigenic stimuli.

Demonstration of Presence of Virus in the Egg. In the absence of characteristic lesions, the only means of recognizing the presence of virus was back-passage into the rabbit. Quicker methods of recognizing infection in the egg were tried unsuccessfully. The supernates of infected membrane suspensions, both unheated and heated for 40 minutes at 70°C[12] were tested for hemagglutination against red cells from the chicken, duck, mouse, guinea pig, rabbit, horse and sheep; but the membranes from infected eggs produced hemagglutination no more regularly than did those of the uninfected eggs used as controls. Precipitin tests and skin tests in rabbits, using as antigen the allantoic fluid of infected eggs yielded negative findings.

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† Tumor tissue or testicle removed from a rabbit at the height of infection, *i.e.* about 6 days after inoculation, is usually infectious when diluted 10^{-5} or even 10^{-6}. Thus the titer obtained with egg passage virus even under optimal conditions is not quite so high as in the case of rabbit passage virus.

[8] Lush, D., *Australian J. Exp. Biol. and Med. Sc.*, 1937, **15**, 131.

[9] Haagen, E., and Du Dscheng-Hsing, *Zentr. Bakt.*, 1938-39, **143**, 23.

[10] Hurst, E. W., *Australian J. Exp. Biol. and Med. Sc.*, 1938, **16**, 205.

[11] Ahlstrom, C. G., *J. Path. and Bact.*, 1938, **46**, 461.

[12] Mills, K. C., and Dochez, A. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 140.

Identification of the Virus Obtained upon Egg Passage. That the virus passed in the egg was actually fibroma virus was shown in 3 ways.

(a) Rabbits inoculated on different occasions with egg passage virus were challenged 2 to 3 weeks later with glycerinated rabbit passage virus and to this they were found to be immune.

(b) A rabbit was immunized by subcutaneous and intratesticular inoculation of egg passage virus and another rabbit was similarly immunized with rabbit passage virus. Both were bled after 14 days and their respective sera used in a neutralization test against fresh rabbit passage virus. Both sera neutralized the virus, no lesion appearing where the mixture was inoculated, whereas areas injected with control mixtures of virus and normal rabbit and virus and saline displayed tumor formation.

(c) A rabbit injected with 8th egg passage fibroma virus was challenged 6 weeks later with a subcutaneous injection of rabbit passage myxoma virus. A control rabbit receiving the same inoculum was dead of myxoma in 10 days, whereas the rabbit which had previously been exposed to egg passage fibroma virus showed no signs of illness. In view of the regularity with which myxoma, virus causes death in normal rabbits it seems certain that the inoculation with egg passage fibroma virus had protected the rabbit against an otherwise fatal infection with myxoma, a phenomenon first described by Shope[7] in connection with rabbit fibroma.

Growth of the IA Strain of Virus in the Egg. Only 2 experiments were carried out with the IA strain of virus. In both cases the virus was lost at the 4th passage, owing to bacterial contamination. No lesions were observed in the infected eggs. In the one case in which the virus titer was determined it was only 10^{-2} at the 3rd passage.

Discussion. The experiments described here show that at least one strain (the OA strain) of the virus of infectious fibroma of rabbits can readily be propagated on the chorioallantoic membranes of the developing hen's egg. Although the titers of virus obtained from infected membranes compared favorably with these from rabbit tumor tissue, the virus did not, in our experience, show any tendency to invade the embryo and was not demonstrable in the extra-embryonic fluids. Likewise when virus propagated for a number of passages on the chorioallantoic membrane was injected into the yolk sac it rapidly disappeared. It is possible that virus which had been subjected to a greater number of passages on the chorioallantoic membrane might display greater invasiveness for the egg.

Passage through the embryonated egg did not seem to alter the type of lesion for its original rabbit host. This finding was rather unexpected, in view of the fact that the virus did not produce in the egg the pathological changes which are characteristic in the rabbit, *i.e.* intense fibroblastic proliferation with production of tumor-like masses and, in the wild cottontail rabbit, very striking cytoplasmic inclusion bodies in the overlying epithelium. No such bodies could be found in epithelium covering the chorioallantoic membrane.

Summary. 1. The virus which is the causative agent of infectious fibroma, giving rise to tumor-like nodules in rabbits, was maintained by serial passage on the chorioallantoic membrane of the embryonated hen's egg for 18 consecutive passages.

2. The titer of virus in infected chorioallantoic tissue was slightly lower than the titer in rabbit tissues. It was maintained by using eggs incubated for 11 days prior to inoculation and making passages at 3-day intervals.

3. Invasion of the embryo and extra-embryonic fluids did not take place in eggs inoculated on the choriallantoic membrane. Inoculation of the virus into the allantoic sac, the amniotic sac, the yolk sac and the embryo itself yielded negative results.

4. Although marked edema of the chorio-allantoic membrane was noted in some of the infected, no pathognomonic lesion, gross or microscopic, was present, so that it was necessary in every instance to resort to back-passage into rabbits to demonstrate the presence of virus.

5. The type of lesion produced in rabbits by the virus was not significantly altered by repeated passage in hen's eggs.

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Effect of Cholecystectomy on Fecal Fat Excretion in Dogs.*

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In a previous report[1] it was demonstrated that diversion of bile from the intestine in dogs results in the failure of absorption of about 60% of fat ingested in the diet. It was also found that oral administration of various bile salt preparations failed to correct this steatorrhea.[2] One of the questions raised by these results concerned the possible role in fat absorption of the manner of entry of bile into the intestine. It was of interest, therefore, to determine whether cholecystecomy, which converts the normal intermittent flow of concentrated bile into a continuous flow of dilute bile into the intestine,[3] would result in a defect in fat absorption.

Methods. Eight normal dogs were placed on a daily diet of 335 g of "Pard" with 40 g added lard, making a total fat content of 53 g. After two days on the diet, feces were collected over a 10-day period and pooled for analyses of total fat and free fatty acids[4] and for nitrogen (Kjeldahl). The animals were then cholecystectomized and allowed 2 weeks for recovery. Four animals were then replaced on the original diet while the other 4 were given a higher fat diet consisting of 200 g "Pard" with 70 grams added lard (total fat content, 80 g). Feces were again collected over the last 10 days of a 12-day period, and analyzed. This procedure was next repeated, interchanging the diet for the two groups of dogs. Finally the dogs were sacrificed and autopsy revealed complete removal of the gallbladder and moderate dilation of the biliary passages.

Results. The mean daily excretions of fat and nitrogen in the feces of 8 dogs both before and after cholecystectomy are presented in Table I. The average total fat excretion of 2.43 g per day compares fairly well with the previously reported value of 3.51 g obtained from 5-day tests on 6 dogs and demonstrated to be independent of the fat content of the diet.[1] Cholecystectomy did not significantly alter this rate of fat excretion. Following the operation the percentage of split fecal fat significantly increased; the meaning of this is obscure since further analysis showed that it occurred only during the first experimental period following cholecystectomy. Fecal nitrogen excretion was unaffected by cholecystectomy, although the lower "Pard" content of the high fat diet resulted in diminished nitrogen excretion, due as previously suggested[1] to the reduced content of crude fiber.

Discussion. The present results clearly indicate that there is no impairment in the absorption of dietary fat or nitrogen subsequent to a change from an intermittent flow of concentrated bile to a continuous flow of dilute bile into the intestine. These results confirm a previous study in which 6 dogs were given

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* Supported in part by a grant from G. D. Searle & Company, Chicago, Ill.

[1] Heersma, J. R., and Annegers, J. H., *Am. J. Physiol.*, 1948, in press.

[2] Heersma, J. R., and Annegers, J. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 339.

[3] Puestow, C. B., *Arch. Surg.*, 1931, **23**, 1013.

[4] Fowweather, F. S., and Anderson, W. N., *Biochem. J.*, 1946, **40**, 350.

TABLE I.
Fecal Fat and Nitrogen Excretion Before and After Cholecystectomy.
Means of 10-Day Tests on 8 Dogs.

Condition	Dietary fat g/day	Total fecal fat g/day		% free fatty acids		Fecal N, g/day	
		Mean	t-ratio*	Mean	t-ratio*	Mean	t-ratio*
Normal	53	2.43	—	68.6	—	.96	—
Cholecystectomy	53	1.96	.99	82.4	2.45†	1.09	2.06
”	80	2.44	.03	80.9	2.47†	.76	4.13‡

* Post-operative tests paired with normal tests.
† Significant at 5 per cent level of probability.
‡ Significant at 1 per cent level of probability.

unspecified amounts of fat and nitrogen over 48-hour test periods before and after cholecystectomy.[5]

Accordingly, in future experimental attempts to correct steatorrhea due to complete bile diversion, the manner of entry of bile preparations into the intestine, whether in continuous or intermittent doses, should not alter the results.

Clinically, these results further imply that bile salt replacement therapy and strict limitation of dietary fat are unnecessary as far as fat absorption is concerned in uncomplicated cases of cholecystectomy.

Conclusion. In 8 dogs studied both before and after operation, cholecystectomy produced no significant change in the daily excretion of fat or nitrogen in the feces.

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[5] Balice, G., *Morgagni*, 1928, **70**, 835.

16644 P

In vitro Antibacterial Action of Extracts from Coptis Root.

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Recent investigations have demonstrated that higher plants are potential sources for antibiotics,[1] and, specifically, extracts of certain Chinese medicinal plants showed bacteriostatic action.[2] In view of the great potentialities of such a finding especially in relation to the study of the nature of action of some of the well known Chinese drugs, a detailed study of the root of *Coptis chinensis*, Franch, belonging to the family of Ranunculaceae,[3] has been made in the past 7 months. The present paper is a preliminary report of the antibiotic activity of the dilute alcohol extract of the coptis root against a number of microorganisms *in vitro*.

Method of Extraction. Samples of coptis root of Szechuan variety were obtained mostly from a well established and reputable Chinese pharmacy, Hsi Ho Nien Tang, Peiping, and some directly from Szechuan. The antibiotic activities of all the samples extracted with the method described below were found to be approximately the same. The macerated dried roots were placed in a large flask with 10 parts of 50% alcohol, and set aside at room temperature for 24 hours. At the end of this period the entire suspension was boiled for 30 minutes and filtered while hot through ordinary filter paper. The clear yellowish amber filtrate was then evaporated to dryness. The dried crude extract was yellowish brown in color and very bitter in taste. Its aqueous solution was used for various tests to be described.

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[1] Benedict, R. G., and Langlyke, A. F., *Ann. Rev. Microbiol.*, 1947, **1**, 193.

[2] Hsü, C. L., *Nat. Med. J. China*, 1947, **33**, 75.

[3] Read, B. E., Chinese Medicinal Plants from Pen Ts'ao Kang Mu, 1936.

Method of Assay. Series of 2-fold serial dilutions of the aqueous extract solution were made in 1% serum broth in small test tubes, and tubes of each series were seeded with one-loopful of 18-hour broth culture of one of the many types of organisms to be tested. The tubes, after being shaken, were incubated at 37°C and readings were made after 24 hours incubation. The end point of bacteriostasis was taken as the highest dilution in which growth in the tubes was completely inhibited and subsequent cultures on blood agar plates still showed growth. Bactericidal power was determined by the absence of growth on subculture on blood agar plates.

Results. The effectiveness of the crude extract of the coptis root in inhibiting cultures of 26 types of bacteria is shown in Table I.

TABLE I.
Inhibition Power of the Crude Extract of the Coptis Root on Bacteria.

Organism	Max. inhibiting dilution
Staph. aureus (2 strains)	1:3200 -1:6400
Staph. citreus (2 strains)	1:6400 -1:12800
Staph. albus	1:12800
Strep. beta hemolyticus	1:3200
Strep. alpha hemolyticus	1:3200
Strep. gamma	1:3200
C. diphtheriae	1:3200 -1:6400
Diphtheroid	1:3200
Klebsiella pneumoniae	1:1600
B. subtilis	1:6400
Micrococcus tetragenus	1:12800
Vibrio comma	1:3200
Shigella dysenteriae (5 strains)	1:400 -1:12800
Shigella sonnei	1:800
Shigella paradysenteriae	1:800
Br. abortus	1:25600-1:51200
Br. melitensis	1:3200
Eberthella typhosa	
H901	0
O901	0
S. paratyphi	0
S. schottmuelleri	0
S. enteritidis	0
E. coli	0
Proteus OX_{19}	0
Pseudomonas pyocynea	0
Serratia marcesecus	0

0 indicates no inhibition in 1:200.

From Table I, it seems very clear that the crude extract of the coptis root in high dilutions inhibited the growth of all Gram positive organisms tested and also that of certain Gram negative ones. The extract appeared particularly effective against *Br. abortus*, as, even in a dilution of 1:25600, growth of this organism was inhibited. Of some interest is that one strain of *Staphylococcus citreus*, isolated from a patient with carbuncle, and highly resistant toward the action of penicillin (50 U/ml) showed great susceptibility to the crude extract in the dilution of 1:12800. When subcultures were made on blood agar plates, it was shown that the bacteriostatic power of the extract was usually 2-4 times greater than its bactericidal power.

Tests for Toxicity. Adult albino rats of 200 g body weight were injected intraperitoneally with 5% aqueous extract solution and observed at intervals of 10 minutes, 30 minutes, 1 hour and 24 hours after injection. Amounts of 2 and 1.5 ml caused death of the animals in 30 minutes, while those of 1 and 0.5 ml produced no obvious toxic symptoms. The animals remained well in 1 week.

Discussion. The root of coptis has been generally used as a bitter tonic, but, in China, it has been extensively used in the treatment of dysentery since ancient times.[4] Results of *in vitro* tests made in the present study extended those of the previous worker in demonstrating bacteriostatic power against various types of dysentery bacilli and may explain why coptis root has been used empirically by the Chinese. The unexpected finding of the special effectiveness of this extract against our stock strain of *Br. abortus* but not particularly so against *Br. melitensis* requires further study. It may be mentioned that the active principle so far has been found to be completely adsorbed on activated charcoal. Further investigations regarding purification and chemical analysis and *in vivo* tests of this extract are being undertaken.

Summary. The dilute alcohol extract of the coptis root has been demonstrated to have a definite and fairly high antibacterial action against a number of both Gram positive and Gram negative bacteria. Its lethal dose for albino rats has been found to be more than 250 mg per kg body weight.

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The author wishes to express his deep gratitude to Professor Samuel H. Zia for his constant advice and helpful criticisms during this investigation.

16645

Anticoagulant Effect of Dicumarol at Various Prothrombin Levels in Dogs.

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(Introduced by I. S. Ravdin.)

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Since the introduction of dicumarol for clinical use[1] there have been differences of opinion concerning the prothrombin reduction required to produce an adequate anticoagulant effect. Some investigators recommend that the prothrombin level be reduced to 10 to 30% of normal.[2,3,4] Others state that the effective therapeutic prothrombin zone lies between 20 and 60%;[5] 35 and 50%[6] and 50 and 60%.[7]

In 2 reported series of patients in which the prothrombin was reduced to 10 to 30% of normal, hemorrhage occurred in 5%[3] and 6%[4] of the patients. Excessive hypoprothrombinemia (below 10%) occurred in 10 to 20% of patients following doses of dicumarol which usually resulted in 10 to 30% prothrombin levels.[4] It seems probable, therefore, that the maintenance of higher prothrombin levels might be preferable if the anticoagulant effect is adequate at higher levels.

Little experimental work has been done to clarify this problem. In the experiments herein reported the anticoagulant effect of dicumarol at various prothrombin levels in dogs has been studied in order to determine whether there is a definite and consistent correlation between prothrombin levels and anticoagulant activity.

Method. The dogs were anesthetized with intravenous sodium pentobarbital, and the femoral artery was exposed and cannulated with as little trauma as possible. Thrombosis was produced by inserting into the femoral artery a sterile glass cannula 1.0 mm in diameter at its constricted opening. The cannula widened out to a 3.5 mm diameter where it was connected to a mercury manometer by sterile rubber tubing. The entire system was filled with sterile physiological saline solution. Additional saline was introduced through a T side-arm in the tubing to produce a positive pressure approximately equal to the mean arterial blood pressure in order to prevent the passage of blood into the tubing. When thrombosis occurred, the mercury column in the manometer ceased oscillating, and this was taken as the end-point.

The prothrombin level of each dog was determined within two hours of the time it was cannulated by the Quick method, with the plasma diluted 50% with normal physiological saline solution. A normal curve was plotted each day the experiment was performed to eliminate errors due to a variation in the potency of the thromboplastin. Table I expresses the prothrombin times (in seconds) of various percentages of normal plasma (50%

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[2] Barker, N. W., *Minnesota Med.*, 1944, **27**, 102.

[3] Allen, E. V., *J.A.M.A.*, 1947, **134**, 323.

[4] Cosgriff, S. W., Cross, R. J., and Habif, D. V., *Surg. Clin. N. Am.*, April 1948, 324.

[5] Levan, J. B., *Ann. Int. Med.*, 1946, **25**, 941.

[6] Peters, H. R., Guyther, J. R., and Brambel, C. E., *J.A.M.A.*, 1946, **130**, 398.

[7] Cummine, H., *M. J. Australia*, 1947, **34**, 302.

TABLE I.
Plasma Prothrombin Times (in Seconds) of Normal Healthy Dogs.

Percentage dilution of normal control plasma (50% diluted)	Total No. of determinations	Avg time (sec)	Range of times (sec)
100	95	9.9	8.2-11.5
90	18	10.2	9.0-11.5
80	21	10.3	9.0-11.8
75	35	10.8	9.4-12.9
60	31	11.0	9.6-13.9
50	55	11.3	9.8-14.8
40	39	12.4	10.0-17.6
30	43	14.0	10.5-19.8
25	36	14.7	12.7-21.6
20	51	17.1	12.3-26.1
15	33	18.7	15.3-32.3
10	47	27.6	18.2-50.7
5	8	38.0	35.9-41.0

diluted) and shows the averages and ranges obtained.

Results. In a control series of 14 normal animals the femoral arteries were cannulated 22 times. Thrombosis occurred, on the average, in 6.3 minutes, with a variation from 3 to 10 minutes (Fig. 1).

The experimental series consisted of 32 dogs ranging in weight from 8.0 kg to 14.2 kg. Dicumarol in doses from 10.0 mg to 400 mg was administered orally 72 hr before the experimental procedure. Prothrombin levels ranging from 95% to 8% of normal were obtained following this therapy.

Prolongation of the "thrombosis time" was found to occur at prothrombin levels of 40% and below (Fig. 2). Of 23 dogs with levels below 40%, the average time was 16.6 minutes. In 20 dogs the thrombosis time was prolonged beyond the longest time of any in the control series. In the remaining 3 thrombosis occurred (10, 10 and 9 minutes) at about the same time as the longest in the control series (10 minutes). The average prolongation of the thrombosis time did not vary significantly at 10, 20, 30 and 40% prothrombin levels.

Prolongation of the "thrombosis time" was not found to occur in dogs with prothrombin levels of 44% or higher. All of the nine animals in this group had "thrombosis times" within the limits of the controls, the average being 7.2 minutes.

In 10 of the dicumarolized animals with prothrombin levels from 8 to 95%, there was no correlation between the Lee-White coagulation time and the cannula "thrombosis time".

Comment. Advantages of the method used are that: (1) the endpoint is clear cut; (2) thrombosis occurs in a relatively short period of time; and (3) the time of occurrence of thrombosis in control animals is relatively constant. Since thrombosis occurred in or about the cannula, this is not strictly an *in vivo* method of producing throm-

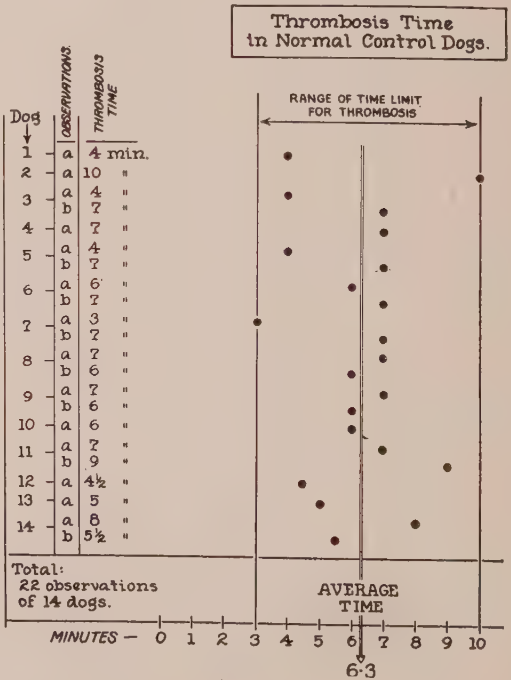

FIG. 1.

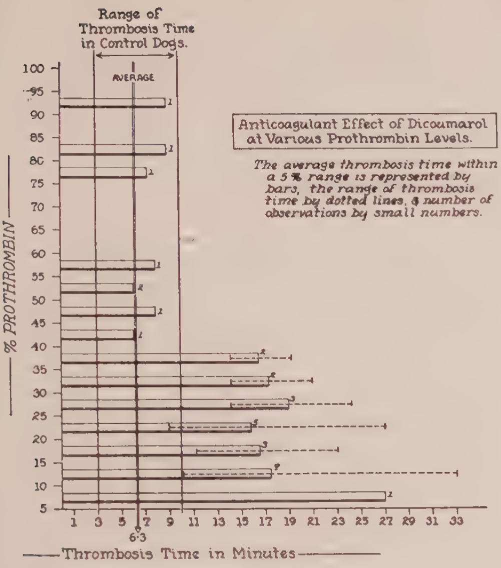

FIG. 2.

bosis, as compared with clinical thrombosis. Neither is it an *in vitro* method, as shown by the lack of correlation between the Lee-White coagulation time and the cannula "thrombosis time". For the purpose sought in this experiment of obtaining a comparative quantitative estimate of the anticoagulant activity of dicumarol at various prothrombin levels, the method has appeared adequate. The method of producing thrombosis used in this experiment obviously does not simulate clinical conditions in which thrombosis occurs. A more suitable method of producing experimental thrombosis has not been devised so far as we know.

The two striking results in the experiment are: (1) the sharp line of demarcation of anticoagulant activity at a prothrombin level of 40% and (2) the apparent uniformity of the average anticoagulant activity at 10, 20, 30 and 40% prothrombin levels.

It is to be emphasized that under the conditions of these experiments the response to dicumarol in dogs may bear little relationship to dicumarol response in man. Yet it is apparent that in dogs: (1) prothrombin levels above 40% had no demonstrable effect upon prolongation of the thrombosis time and (2) prothrombin levels below 40% did significantly increase the thrombosis time in 20 of 23 experiments.

In view of these results and the lack of conclusive experimental evidence elsewhere, the question must be raised as to whether the clinical use of prothrombin levels of 10% to 30% may be too low or whether levels of 40% to 60% may be too high for effective yet safe anticoagulant therapy.

As to the apparent uniformity of anticoagulant activity at 10, 20, 30 and 40%, this may merely reflect a limitation of the sensitivity of the method or else may reveal that an all or none, rather than a mass action phenomenon, is associated with the method. This cannot be definitely answered at present from the data available.

Conclusions. 1. The anticoagulant effect of dicumarol at various prothrombin levels in dogs has been studied.

2. In control animals thrombosis occurred, on the average, in 6.3 minutes, with a variation from 3 to 10 minutes.

3. In dicumarolized dogs the time of thrombosis was not delayed at prothrombin levels above 40%. Below 40% thrombosis occurred on the average, in 16.6 minutes.

16646

Response of the Duodenum to Morphine.

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The earliest investigators concerned with the response of the intestine to morphine were predominantly of the opinion that this drug produced a relaxation of the muscle of the intestine. More recent investigations have changed this trend of belief by showing that morphine produced an increased pressure within the intestine and increased movements of various segments of the intestine as measured by one or more forms of a balloon method.

It has been pointed out by Krueger[1] and emphasized by Quigley, Highstone and Ivy[2] that erroneous conclusions may be drawn concerning the sum total effect of a drug on the intestinal muscle by using a method which involves inserting a balloon in the lumen of the intestine and recording activity in terms of changes in volume of the balloon. Thus in an attempt to improve the experimental procedure, bolus methods[2] and multiple balloon methods[3] have been devised and used.

The present study is concerned with the muscular response of the duodenum to morphine as measured by a method which gives simultaneous graphic recordings of both the circular and longitudinal muscle activity in a given segment of the duodenum in the intact anesthetized dog.

Methods. The circular contractions of the duodenum were recorded by the method of Krop and Loomis.[4] The balloon inserted in the duodenum was constructed so that its length was not more than 2 cm. Such a balloon would come in contact with about 1 cm length of intestinal mucosa when fully expanded. Observations with this apparatus *in situ* showed that longitudinal movements of the intestine did not record through the manometer. Circular contractions, on the other hand, compressed the balloon and were recorded as changes in the level of the fluid in the manometer. The longitudinal contractions of the duodenum were recorded by the internal organ apparatus described by Jackson.[5] Observations with this apparatus *in situ* showed that the records obtained were predominantly those of longitudinal muscle activity.

Mature mongrel dogs in good health were used throughout the experiments. The dogs were anesthetized with 35 mg per kilo sodium pentobarbital given intravenously. A midline abdominal incision from the xiphoid process of the sternum to about 2 cm above the pubis was made. The intestine was retracted to one side, an incision was made in the pyloric end of the stomach on its anterior surface and the balloon was inserted through the incision into the stomach, directed through the pyloric sphincter and into the duodenum so that it lay about 5 cm distal to the sphincter. Jackson's internal organ apparatus was then sutured to the mucosal surface of the segment of duodenum which contained the rubber balloon within its lumen. The intestine was then replaced in its approximate normal position and the cut edges of the abdominal incision were approximated and sutured.

All injections were given intravenously through one of the superficial branches of the femoral vein. The drugs were dissolved in physiological saline. The volume of each injection was always less than 5 cc and the solutions were maintained at a temperature of 38°C. Each injection of a drug was fol-

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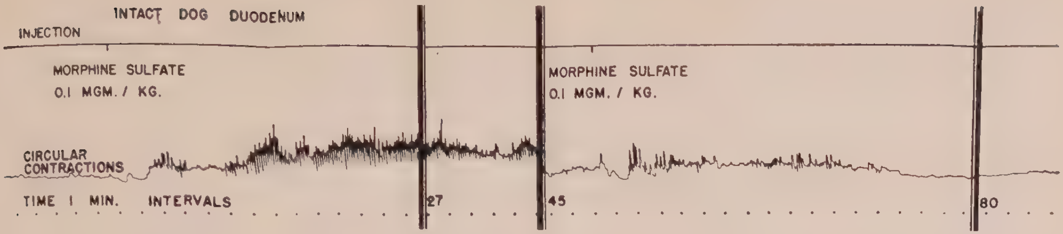

Fig. 1.

The effect of repeated equal doses of morphine sulfate on the circular contractions of the duodenum of the anesthetized dog. The drug was injected intravenously at the points indicated on the injection line.

lowed with about 2 cc of physiological saline. Controls using the saline alone showed that the spontaneous intestinal contractions were not altered by this volume of saline.

Results. Early in the progress of the experiments it was found that repeated equal doses of morphine failed to produce equivalent responses in the intestine. Although the muscular responses would be qualitatively similar the intensity and duration of these responses were much diminished with each additional injection. Fig. 1 demonstrates this tachyphylactic type of phenomenon which occurs in the response of the circular muscle when an intravenous dose of morphine of 0.1 mg per kg is given and then repeated after the activity had returned to normal. After this phenomenon had been repeatedly demonstrated all subsequent experiments were conducted so that the animal received only one injection of morphine.

Two principal types of response to the drugs used were obtained. One of the types of response was an increased level of tonus with either an increase or no change in the frequency and amplitude of the normal spontaneous contractions. This type of response is referred to in this paper as "increased activity." The second type of muscular response was a decreased level of tonus and either a decrease or no change in the frequency and amplitude of the normal spontaneous contractions. This type of response is referred to as "decreased activity."

Table I summarizes the gross qualitative responses obtained on a series of experiments conducted on 28 dogs. In all experiments acetylcholine was used as a control drug. The table shows that 0.05 mg per kg of this compound, given intravenously, produced an increased activity of the circular muscle in all but one of 25 experiments. In simultaneous records this type of response occurred in the longitudinal muscle in 18 of the 25 experiments. In one of the experiments both the circular and longitudinal muscle records showed decreased activity. In 5 of the experiments the longitudinal muscle showed decreased activity while the circular muscle responded by an increased activity.

A dosage range of morphine of from 0.01 mg per kg to 1.0 mg per kg was used. The smallest dose of morphine which was consistently effective in producing an alteration in the intestinal muscular activity was 0.05 mg per kg. Fig. 2 shows the type of record obtained. A similarly equivalent qualitative response was obtained in five such experiments. Table I summarizes the response obtained with 0.1 and 0.5 mg per kg of morphine in the series of seventeen experiments on seventeen different dogs. The table shows that the circular muscle responded with an increased activity in 16 of the 17 experiments. In 13 of the experiments the longitudinal muscle showed either no significant response or a decreased activity. With a dose of morphine of 0.5 mg per kg the response of the circular muscle was that of increased activity in all of the 17 experiments. In contrast to this, the longitudinal musculature showed increased activity in only two of the 17 experiments, whereas in 10 of the experiments the result was that of decreased activity and in 5 experiments there was no observable alteration in muscle activity.

One mg per kg of morphine produced an increased activity in the circular muscle in 6 experiments, and in 5 of the 6 experiments a decreased activity was the response recorded

TABLE I.

Summary of Frequency and Type of Response of Duodenal Muscle to Acetylcholine, Morphine, Barium, and Atropine. Maximum and minimum duration of response is presented rather than mean and standard error, since accurate determination of duration of time before the record returned to normal was indefinite within limits of approximately 10 minutes.

	0.05 mg/kg Acetylcholine Longitudinal muscle	0.05 mg/kg Acetylcholine Circular muscle	0.1 mg/kg Morphine SO$_4$ Long. m.	0.1 mg/kg Morphine SO$_4$ Circ. m.	0.5 mg/kg Morphine SO$_4$ Long. m.	0.5 mg/kg Morphine SO$_4$ Circ. m.	1.0 mg/kg Morphine SO$_4$ Long. m.	1.0 mg/kg Morphine SO$_4$ Circ. m.	1.0 mg/kg Barium Chloride Long. m.	1.0 mg/kg Barium Chloride Circ. m.	2.0 mg/kg Atropine SO$_4$ Long. m.	2.0 mg/kg Atropine SO$_4$ Circ. m.
1. No. of experiments	25	25	17	17	17	17	6	6	5	5	3	3
Response: (number of exper. showing:)												
2. Increased activity	18	24	4	16	2	17	0	6	5	5	0	0
3. Decreased activity	5	1	6	1	10	0	5	0	0	0	3	3
4. No significant response	2	0	7	0	5	0	1	0	0	0	0	0
5. Range of duration of response (min)	2-16	2-9	40-110	40-100	30-160	40-160	60-200	60-280	—	—	—	—

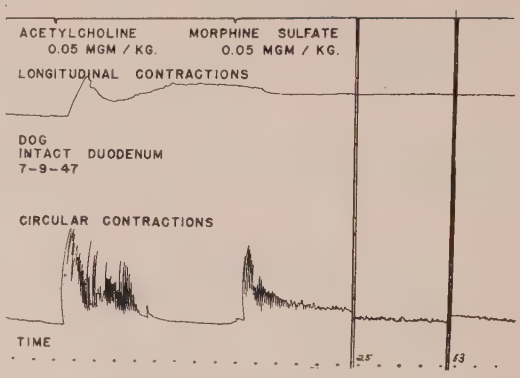

FIG. 2.

The "increased activity" of the circular muscle and simultaneous "decreased activity" of the longitudinal muscle produced by 0.05 mg per kg of morphine sulfate following a control acetylcholine injection. Time is recorded as dots at one minute intervals.

by the longitudinal muscle apparatus. Fig. 3 is a typical record of this result.

These results show that the type of response was essentially independent of the dose of morphine used over the range which was studied. The duration of the response was similar in both the circular and longitudinal groups (Table I) for a given dose of morphine. The duration of response generally was greater with higher doses than with the lower doses over the range studied.

Barium chloride in a dose of 1.0 mg per kg consistently produced an increased activity in both the circular and longitudinal muscle records. When morphine is given at the time when a maximum response to barium is present generally little or no alteration in the activity of the duodenal muscle occurs.

Atropine repeatedly produced a decreased activity in both the circular and longitudinal muscle of the duodenum. A dose of morphine of 0.1 mg per kg produced an increased activity in the circular muscle in the atropinized animal at a time when a control acetylcholine injection produced no response. The response of the longitudinal musculature in an atropinized animal was indefinite since atropine frequently produced such a profound decreased tonus and often completely abolished all activity of the muscle. However, when slight activity remained in the longitudinal muscle after administration of atropine, it was abol-

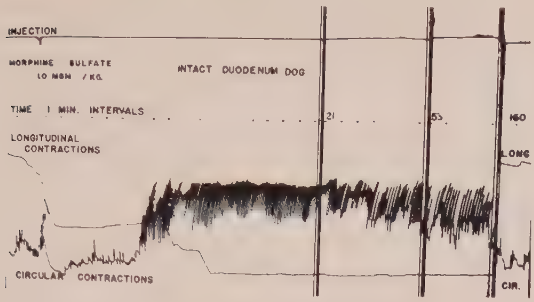

FIG. 3.
The "increased activity" of the circular muscle and the simultaneous "decreased activity" of the longitudinal muscle of the intact segment of the dog duodenum as it is produced by a dose of 1.0 mg/kg of morphine sulfate.

ished by the subsequent administration of morphine.

Atropine only partially decreased the increased activity in the circular muscle produced by morphine. Atropine also abolished any activity present in the longitudinal muscle group when given following the injection of morphine.

In 3 experiments in the physostigminized animal, morphine produced a response of a type similar to that most frequently produced in the normal animal. This was a decreased activity of the longitudinal muscle and an increased activity of the circular muscle (Fig. 7). However, the mean duration of the response to morphine (0.1 mg per kg) in the eserinized animal was 222 minutes (3 experiments) whereas the maximum duration of response obtained in the normal dog to 0.1 mg per kg of morphine was 110 minutes (17 experiments).

Discussion. The evidence in the literature at the present time indicates that morphine does not produce a consistently specific type of effect on smooth muscle throughout various parts of the gastrointestinal tract.[1–3,6–9,11–13] Furthermore, reports concerned with the effect of morphine on the atropinized or eserinized intestine are not consistent in the same or different animals.[7,10,14]

Acetylcholine and barium chloride consistently produced increased activity in both the circular and longitudinal muscle layers with the experimental arrangement described in this paper. Atropine sulfate consistently produced decreased activity of both of these muscle layers. The most consistent response produced by morphine injected intravenously was that of an immediate increased activity of the circular muscle layer and a decreased activity of the longitudinal muscle layer. This type of response was marked and prolonged and records always showed a return to normal when allowed sufficient time. Occasionally there occurred an immediate very brief (5 minutes or less) interval of little change or an increased activity of the longitudinal muscle or a decreased activity of the circular muscle following the injection of morphine.

Summary and conclusions. 1. The phenomenon of tachyphylaxis of the intestinal musculature to morphine was demonstrated.

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2. The most consistent response of the circular muscle of the duodenum to morphine was an increase in the many phases of muscular activity. This includes elevation of the tonus level with increased frequency and amplitude of the normal spontaneous tonic contractions.

3. The most frequent though not completely consistent response of the longitudinal muscle was found to be either a decrease or an abolition of the many phases of the muscle activity. This includes a depression of the tonus level with a decreased frequency and amplitude of the normal spontaneous tonic contractions.

4. The type of response of the duodenum to morphine was independent of the original state of tonus and independent of the dose over the range of 0.01 mg to 1.0 mg per kg.

The technical assistance of Mr. Herbert Stowe is gratefully acknowledged.

16647 P

Protective Action of Dibenamine After Hemorrhage and After Muscle Trauma.*

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Wiggers and associates[1,2] have demonstrated that dogs given the sympatholytic substance Dibenamine showed a higher survival rate than controls from shock produced by hemorrhage. The inference made is that abolition of a severe vasoconstriction normally following hemorrhage protects the life of the animal.

In a series of dogs under morphine-nembutal anesthesia, 6 arterial hemorrhages of 5 cc/kg b.w. each, were performed at 10 minute intervals. Aortic pressure pulse contours were recorded during the hemorrhage period, and for an additional hour. All wounds were then closed, and the animals returned to their cages. Cardiac outputs were calculated from the pressure pulse contours,[3] and in a selected series by the dye injection method,[4] and vasomotor resistance (Rv) calculated as the mean aortic pressure less 20 mm Hg divided by the flow per second.[5] Specific gravity values of plasma and whole blood were determined at 10 minute intervals by the falling drop technic.

This experiment was done during the summer months, so that an expectedly sub-lethal hemorrhage proved fatal in 13 of 14 dogs, with an average survival of the 13 dogs of 4 hours. Ten more dogs were given 10-20 mg/kg Dibenamine,† in 50 cc saline, *i.v.*, one hour before the hemorrhage was begun. The completeness of the sympathetic block was demonstrated by the injection of epinephrine 5 minutes before the first bleeding. Nine of these animals survived. To rule out the possible effect of the salt solution itself, the last 5 of the control series received 50 cc saline one hour before the hemorrhage. All of these dogs died.

Systolic and diastolic pressure values for the two groups were not significantly different The Dibenamine treated animals showed slightly higher blood flow values throughout, with faster pulse rates. Vasomotor resistance values, while showing roughly parallel changes in the two groups, were consistently lower in the treated dogs. There was no demonstrable correlation between the dilution of the blood and the survival period. In fact, as a group, the treated animals showed less dilution than the controls.

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* This research was aided by a grant from the Life Insurance Medical Research Fund.

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† We are indebted to Dr. W. Gump of Givaudan-Delawanna, Inc., for the Dibenamine Hydrochloride used in this study.

It has been shown that animals subjected to contusion of the muscles of both hind legs, by multiple blows with a light mallet, show higher resistance values than after hemorrhage,[6] and die after a smaller blood volume loss than would be required by hemorrhage.[7] This higher resistance is presumably brought about by afferent nervous discharge from the traumatized limbs.[8] Nine control animals, under morphine-nembutal anesthesia, were given 80 blows/kg b.w. to each hind leg. All these dogs died, with the average survival of 3½ hours. Nine more dogs were given Dibenamine, as above, 1 hour before the trauma. Eight of these recovered, the ninth dog surviving 15 hours. As with the hemorrhage experiment, pressure values were not different in the two series. Pulse rates of the Dibenamine-treated animals were greater, and flows were maintained at higher levels. They therefore showed a lower vasomotor resistance.

A limited number of blood volumes, determined by T-1824 injection, indicated that the blood volume loss in these animals was sufficient in itself to have caused death. The blood volume loss was of similar magnitude in treated and untreated dogs. Peripheral resistance was definitely higher than after hemorrhage. The treated dogs did not show this rise in resistance, but rather a vasodilation. Death in the control series may be preceded by the appearance of cardiac irregularity, which developed while the peripheral resistance was still high. Both groups showed a hemoconcentration. In the control series, however, this developed not during the trauma but after its completion.

Summary. A hemorrhage of 30 cc/kg proved fatal in 93% of the cases. Pretreatment with Dibenamine reduced this mortality to 10%. Pulse rate and blood flow per second were consistently greater in the second series. Administration of 80 blows/kg to each hind leg with a light mallet produced fatal shock in 100% of the cases. Pretreatment with Dibenamine reduced the mortality to 11%. Control animals showed vasoconstriction, treated animals vasodilation.

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16648

Is Mannitol Metabolized?*

John Kapp Clark† and Harold G. Barker.‡ (Introduced by Carl F. Schmidt.)

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If, as has been recently suggested,[1,2] mannitol is in part disposed of by non-renal means, probably metabolic, it cannot be used as a measure of extracellular fluid space.[3] Such a circumstance would render invalid calculations of glomerular filtration rate in which the rate of infusion is substituted for the rate of excretion, UV, in the clearance formula $C = UV/P$ after the plasma level, P, has become constant,[4] or from the falling curve of plasma concentration without urine collection,[5] if mannitol is used as a test substance. Since the publication of the constant infusion technic for determining renal clearances,[4] we have routinely infused our test solutions, mannitol and para-aminohippurate, from a syringe with a motor driven worm screw, and our data on subjects with relatively normal renal function, in cases where both infusion solutions and urine were analyzed, indicate no extra-renal loss when mannitol recovered in the urine is balanced against that infused. In these tests, a period of one hour, when possible, was allowed for equilibration, urine was collected by catheter with bladder washing and clearance periods were usually 20 minutes or longer. Plasma levels varied from 100 to 50 mg%. Mannitol was determined by a modification[6] of Smith's method[7] in most cases and by the colorimetric method[8] in a few. Total para-aminohippurate was determined by a modification of Smith's method.[9]

Results. Our data are presented in two groups. The first, the unselected series, consists of all patients in whom both urine and infusion solutions were analyzed and contains 92 clearance periods in 29 tests on 22 patients with relatively normal renal function.§

The average urinary recovery of mannitol for all 92 periods was 100.4 mg for every 100 mg injected (range 62 to 156). The standard deviation of this series is 16.7; standard error of the mean 1.74. For the difference 100.4-100.0 the t value is 0.23, indicating that the amount recovered in the urine does not differ significantly from that injected.

In many of the clearances satisfactory mannitol plasma equilibrium was not achieved due to inaccurate preclearance estimation of renal function, a priming dose of improper size, changing renal function, etc. In addition, some periods are inaccurate due to technical difficulties such as obvious urine collection errors, chemical analytical errors, pump

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† National Institute of Health Senior Research Fellow and formerly Research Fellow in Pharmacology.

‡ Harrison Fellow in Surgical Research, and formerly Research Fellow in Pharmacology.

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§ Mimeographed sheets containing data will be furnished on request.

failures, etc. These cases could be excluded, although the fact that their inclusion introduces no significant in-out difference indicates that random rather than systematic errors are involved.

The second group of data, the selected series, consisting of clearance periods chosen on the basis of plasma equilibrium and technical perfection, but, of course, without regard to mannitol recovery, contains 30 periods from 16 tests on 12 patients.[§]

This series contains only periods preceded and followed by determinations of plasma concentration that did not differ by more than 3.5 mg%, a value within the error of the method (standard deviation = ±2.5 mg%). An actual difference of this magnitude, in 2 specimens drawn 30 minutes apart, would represent, on the basis of an average normal mannitol space of 15 liters,[3] a gain or loss of 17.5 mg/min. and thus might obscure a very large extra renal loss. To determine whether equilibrium had been obtained in these periods, they are further subdivided into 3 subgroups: those periods with bracketing plasma levels differing by less than 1 mg%, those with levels falling 1 to 3.5 mg%, and those rising 1 to 3.5 mg%. If equilibrium had not been established one would expect an excessive urinary recovery in the periods with falling levels and a deficient recovery in those with rising levels. Eighteen periods were bracketed by levels differing by less than 1 mg% and the mean out-in ratio of these is 0.990. There were 8 periods in the falling group with a mean ratio of 0.995 and 4 in the rising group with a mean ratio of 0.970. Since, if these values represented actual differences in plasma concentration, the ratios would be 117 and 0.82 for the falling and rising groups respectively we feel that equilibrium had been established. In these selected periods, an average of 99.3 mg of mannitol was excreted for every 100 mg injected (range 87-114). This small series has a standard deviation of 6.0 with a standard error of the mean of 1.1, giving for the difference 100.0-99.3 a t value of 0.64, thus failing to establish a difference between the urinary recovery of mannitol and the amount injected.

Summary and Conclusions. 1. In 92 unselected clearance periods in 29 tests on 22 patients 100.4 mg of mannitol were recovered in the urine for every 100 mg injected.

2. In 30 periods in 16 tests on 12 patients selected on the basis of plasma equilibrium and technical adequacy 99.3 mg of mannitol were recovered for every 100 mg injected.

3. These data reveal no significant extrarenal disposal of mannitol.

16649

Twelve-hour Nocturnal Gastric Secretion in Uncomplicated Duodenal Ulcer Patients: Before and After Healing.

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A recent study[1,2] demonstrated that the volume, concentration, and output of acid in the nocturnal gastric secretion are usually higher in patients with duodenal ulcer than in normal healthy individuals. The patients had been admitted to the hospital because of ulcer distress. Several observers have suggested that the presence of symptoms may be a contributing factor in causing hypersecretion in duodenal ulcer patients. It

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TABLE I.

Twelve-Hour Continuous Nocturnal Gastric Secretion in Patients with Duodenal Ulcer Before and After Healing.

	Uncomplicated active duodenal ulcer				Uncomplicated healed duodenal ulcer			% change		
Case	Vol. (cc)	Free acid (Cl units)	Free HCl (mg)	Time interval (mo.)	Vol. (cc)	Free acid (Cl units)	Free HCl (mg)	Vol.	Free acid (Cl units)	Free HCl (mg)
1	1104	53	2130	6½	857	22	689	0	—40	0
	737	51	1348		979	39	1375			
2	581	49	1035	4½	770	42	1163	+44	—	+
	494	52	933		778	35	991			
3	1466	103	5481	3	1324	82	3954	0	—	—
	1102	115	4606		1128	94	3869			
4	718	13	333	3	818	18	536	+	0	+ 27
	680	21	504		720	20	524			
5	1398	57	2887	3	1430	60	3123	0	0	+
	1098	67	2657		1358	75	3707			
6	643	55	1290	3	349	25	320	—52	—44	— 74
	614	52	1162		258	34	317			
7	675	62	1523	30	1019	47	1726	+53	0	+110
	630	40	917		1164	74	3122			
	840	40	1223							
8	1800	65	4259	9	1623	64	3787	0	0	0
	1700	55	3403							
	1650	62	3724							
	1600	48	2795							
	1560	57	3237							
9	940	112	3828	1½ yr	1176	92	3933	0	0	0
	1396	97	4921		1108	75	3044			
	1262	70	3211							
10	1200	58	2533	2¾ yr	1344	56	2726	0	0	0
	1400	55	2803		1394	63	3033			
	1500	61	3330							
	2000	34	2476							
11	543	42	830	2 yr	731	47	1251	+	+	+ 28
	621	50	1130		654	53	1262			
12	525	34	650	2½ yr	1039	16	618	+74	0	0
	650	20	473		897	23	767			
	500	6	109							
13	939	45	1553	7	1109	37	1515	+	—	0
					1032	42	1584			

+ Slight but not significant increase.
— Slight but not significant decrease.

TABLE II.
Nocturnal Gastric Secretion: Average Volume, Free Acidity, and Output Free Hydrochloric Acid.

	Vol. (cc)	Free acid (Cl units)	Mg free HCl
Normal	581	29	661
Duodenal ulcer (series No. 1)*	1004	61	2242
Active uncomplicated duodenal ulcer (present series)	1047	58	2208
Healed uncomplicated duodenal ulcer (present series)	1002	54	1957

* For complete data see reference No. 2.

seemed desirable therefore to measure the nocturnal gastric secretion in a group of patients with uncomplicated active duodenal ulcer having distress, and to repeat the same determinations in the same individuals during a period when the ulcer was healed and symptoms were not present.

Methods. The 12-hour nocturnal gastric secretion was measured in 13 patients with duodenal ulcer during a period when their ulcer was easily demonstrable roentgenologically and when typical distress was present. The studies were repeated in the same individuals after medical treatment had led to healing of the ulcer roentgenologically and to the complete subsidence of symptoms; the latter studies were carried out 2½ months to 2¾ years after the ulcer was known to have healed.

The methods of study were identical with those described previously,[1,2,3] enabling a strict comparison with our earlier observations. All data were submitted to statistical analysis. Previous studies have shown[2] that in the same individual with duodenal ulcer the total volume of the nocturnal gastric secretion on the average varies by approximately 20% and the free acidity and output of free hydrochloric acid by approximately 25%. This spontaneous variation was taken into account in evaluating the present results.

Results. The data for total volume, free acidity and output of free hydrochloric acid in the 12-hour nocturnal gastric secretion in the same individual during the phase of active ulcer and again during the period of healed ulcer are presented in Table I. The volume was unchanged in 9, significantly greater in 3, and significantly diminished in 1 (Case 6). The free acidity of the total volume was unchanged in 11, and diminished in 2 (Cases 1 and 6). The output of free hydrochloric acid was unchanged in 9, significantly greater in 3, and diminished in 1 (Case 6). For the entire group there were no significant differences in the average volume, free acidity and output of free hydrochloric acid before and after healing of the ulcer (Table II).

The present data, in agreement with that obtained in previous studies[1,2,3] (Table II), indicate that the average nocturnal gastric secretion in patients with uncomplicated duodenal ulcer and also in patients with healed uncomplicated duodenal ulcer is significantly greater than that of normal healthy individuals. Furthermore, the average night secretion of patients with healed uncomplicated duodenal ulcer is not significantly different from that of patients with duodenal ulcer requiring hospitalization because of distress (Series I).[1,3]

The hourly pattern of acid secretion after the ulcer had healed was the same as had been noted during the period of active ulcer. The secretion of acid in all individuals in this series was continuous and was maintained at a relatively higher level than is seen in normal individuals. In no instance were there periods of anacidity for as long as one hour.

The persistence of hypersecretion in the vast majority of cases after healing of duodenal ulcer emphasizes the importance of continued careful antacid therapy in such patients.

Conclusions. 1. In the same patient with duodenal ulcer the 12-hour nocturnal gastric secretion is usually unaltered with healing of the ulcer.

2. The *average* 12-hour nocturnal gastric secretion of patients with active ulcer is not

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[3] Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Gastroenterology*, 1948, **10**, 939.

significantly different than in patients with healed duodenal ulcer.

3. Twelve-hour nocturnal gastric secretion in patients with healed duodenal ulcer and who are without symptoms is significantly greater than that of normal healthy individuals.

16650

Can Gonadotrophic Hormones Cross Two Parabiotic Unions in Rats?

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It has been shown that when 3 rats are joined in parabiosis gonadotrophic hormones produced by the middle member of the triplet set affect both of the other rats at the same time.[1] The present experiment was designed to test the ability of gonadotrophic hormones to pass from one lateral member, through the middle animal, and into the opposite lateral triplet of 3 united rats. Preliminary results, reported in abstract form,[2] have been extended and confirmed.

Methods and Results. Results are given for 19 sets of triple parabionts. Eight sets were united on the 25th day of life and 11 on the 40th day. A total of 14 single females, littermates of the parabionts, were used as controls in the two groups. All animals were autopsied at the age of 50 days. The results for the 2 age groups substantiate each other.

In producing triple parabiosis the edges of the abdominal walls were sutured together without establishing a coelio-anastomosis. The scapulae were held together by 3 interrupted sutures. The skin was joined by metal wound clips from ear to base of tail after removing strips of skin from the adjacent sides of each animal.

Two females from the same litter were joined in parabiosis, and a castrated male was attached to the left sister to make the triplet set. In all but 2 cases the male was a littermate of the 2 females in the set. The passage of gonadotrophic hormones from the castrated male to the center female was indicated by the enlarged ovaries and uterus and by the involuted thymus (Fig. 1 and Table I). The female to the right, not directly attached to the male, showed no ovarian stimulation, the ovaries weighing even less than those of the littermate controls. The uterus, however, appeared to be slightly stimulated and the thymus very slightly reduced in size. The stimulated ovaries of the middle female contained large follicles and large corpora lutea.

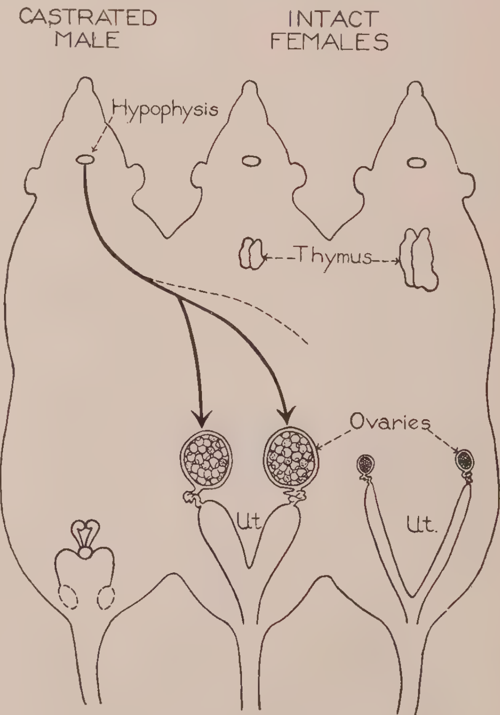

FIG. 1.

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TABLE I.
Some Effects of Triple Parabiosis in Rats—Joined from Days 25 to 50 and from Days 40 to 50.

	Days Joined	No. of Animals	Body wt (g)	Avg organ wt in mg per 100 g body wt		
				Ovaries	Uterus	Thymus
Center female	25-50	8	96	257	333	76
(attached to castrated male)	40-50	11	108	203	264	63
Right female	25-50	8	101	11	200	266
(attached to female)	40-50	11	113	27	170	241
Female controls		6	123	16	95	298
(littermates of parabionts)		8	146	29	152	296

Discussion. In triple parabiotic rats of which the left member is a castrated male, the known ability of gonadotrophic hormones to cross the parabiotic union[3,4,5] is demonstrated by the stimulation of the ovaries of the middle female. The apparent lack of stimulation of the ovaries in the second female suggests that the gonadotrophins are utilized completely by the ovaries of the first female.

The reason for the increase in the weight of the uterus of the second female, in the absence of any enlargement of the ovaries, is not clear. Either there is some transfer of estrogen between the 2 females or the ovaries of the outer female, though reduced in size, are actually elaborating sex hormone. If estrogen from the center female is stimulating the uterus of the other female, a very high concentration of the hormone is indicated since it has been demonstrated that at least 40 times the minimal effective dose must be injected into one member of a pair of ovariectomized females which have been united in parabiosis in order to detect uterine stimulation in the twin.[5] If the small ovaries of the outer female are stimulating its uterus then size of the ovary fails to measure physiological activity.

The slight decrease in the weight of the thymus of the second female is correlated with a proportionate increase in the weight of the uterus and is probably also due to the result of estrogens since gonadotrophins have no direct effect upon the thymus.[6,7]

Summary. Gonadotrophic hormones from a castrated male cross successfully the first parabiotic union to stimulate the ovaries of the middle female of a set of triple parabiont rats. But the gonadotrophins are so completely utilized by the center animal that they produce no detectable ovarian stimulation of the second female.

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16651

Androgen-Induced Growth of the Os Penis of Hypophysectomized-Gonadectomized Rats.*

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It was concluded from earlier experiments carried out by other investigators in this laboratory[1] that under certain circumstances testosterone propionate promoted growth of cartilage and bone in the proximal tibial epiphysis of hypophysectomized rats. In the report of that work it was suggested that explanations for apparent discrepancies noted in the literature dealing with the effects of androgens on bone growth may be found in such variables as age, sex, dosage, length of treatment, etc. In the experiment outlined below a new variable has been introduced in that the bone chosen for study, the os penis, is peculiarly situated in a region that might be expected to be affected profoundly by the deprival or varying supply of androgenic substances. Using normal and castrated rats with intact pituitaries Turner *et al.*[2] showed that testosterone propionate accelerated the development of this bone as judged by skiagrams.

Experimental. It was previously determined[3] that rats of the Long-Evans strain gonadectomized on day 26 and injected daily thereafter for approximately 3 weeks with 0.01 mg of testosterone propionate would show growth of the penis and splitting of the balano-preputial epithelium comparable to that of the normal animal. This dose was therefore assumed to approximate the daily average equivalent of androgen to which the normal animal was exposed during that period of life. As may be seen, then, from Table I some rats hypophysectomized and gonadectomized on day 26 were immediately started on this daily dose of testosterone propionate[†] while others received 0.1 mg. Control animals also doubly-operated on day 26 received 0.1 cc of sesame oil, the volume of diluent used for the hormone.

Terminal normal controls were obtained from the same litters as the experimental rats and kept on the same regime of diet and care. Control tissues from 21- and 26-day-old normal rats as well as from full grown animals were also studied. In this preliminary experiment the rats were allowed to eat *ad lib.*, but it is planned to control the important variable of food-intake in a future experiment in this series. Animals were weighed on days 5, 10, 15, and 21 of the experiment and it was obvious from the appearance of the food cups and the weight gains that the operated animals ate well, although never as much as the normal controls. On the other hand, the normal rats were more active and possessed better body tone.

At necropsy the penis trimmed of skin, and a patella from each animal were fixed in Bouin's fluid, and the operative sites were checked for completeness. After 2 days in Bouin's fluid the os penis becomes decalcified as judged by skiagram, but the tissues were also exposed to 5 parts of concentrated HCl (by volume) to 95 parts of the graded alcohols (50-100%) over another 2-day period. At the 80% alcohol stage the penis was uniformly cut in such a way that the final block of tissue consisted of the distal segment and only the bend of the proximal segment. After the excess fluid was removed from these blocks of tissues they were weighed (see Table I). The penis was cut in dorso-ventral

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* Aided by grants from the Research Board of the University of California.

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TABLE I.
Effect of Testosterone Propionate on the Body Weight, Penis Weight and Length of Os Penis in Rats Castrated and Hypophysectomized on Day 26 and Injected for 21 Days.

No. of rats	Daily dose test prop. mg	Age in days	Avg. wt gain g	Avg. wt distal penis, mg	Avg. length os penis, mm
3†		21			2.73 ± 0.11
3†		26			3.33 ± 0.15
7		47	9	23.5 ± 1.8 (5)*	3.20 ± 0.18 (6)
5	.01	47	5.6	57.3 ± 4.5	3.52 ± 0.14 (4)
8	.1	47	21.2	102.3 ± 4.3 (5)	3.80 ± 0.12
7†		47	103.6	81.5 ± 19.3 (5)	4.1 ± 0.2 (5)

± = Standard deviation.

* Figures in brackets indicate the number of rats (when less than the total) used in a calculation, the penes of those excluded being used for different purposes.

† Normal unoperated controls.

planes in a way that permitted one to obtain representative sections through the greatest length and the central part of the os. Measurements of the bone were made with a calibrated oculo-micrometer. Enough of the patellae were sectioned and stained to provide the information that this cartilage, showing only the earliest signs of ossification on day 26, changes in the 3 weeks after hypophysectomy and gonadectomy even in the untreated rats to a well ossified structure somewhat smaller than in the 47-day-old normal animals.

Results. Table I summarizes the results. It was known from previous experiments carried out in this laboratory that hypophysectomized rats of this age might be expected to gain slightly in weight. The average weight increments of 9 g in 21 days for the operated controls and 5.6 g for the rats on the 0.01 mg level of testosterone propionate are not considered unusual, but the 21.2 g gain with a range from 15-32 g in the 8 rats receiving the higher dose of androgen must be viewed as an effect of the hormone not mediated through the pituitary. Although this gain in weight (1 g per day) parallels that observed in hypophysectomized rats given low dosage of pituitary somatotrophin, it must be considered small (20%) when contrasted with the weight increment in normal control rats of this age. However the penes of the operated rats injected with the 0.1 mg level of testosterone propionate had developed at a slightly faster rate than those of the normal animals; and the average weight of their distal segments exceeded that of the normal rats by 25%. The os penis of the androgen-treated rats, then, may be considered a bone within an organ growing even more rapidly than in the normal animals. The higher dose of androgen permitted the os penis to attain a length approximately 19% greater than the uninjected, operated controls and 92% as great as normal rats of a similar age. The lower dose was less effective as may be seen in Table I. The main histologic findings are represented in the photomicrographs and briefly summarized in the legends.

Discussion. Adequate discussion of these findings must be postponed until further experiments are carried out to determine the possible intermediary role played by organs other than the pituitary (*e.g.*, the thyroid). The growth autonomy of certain parts of the skeletal system as shown by tissue culture experiments[4] and as re-emphasized by the Dutch investigators who witnessed bone growth in rats hypophysectomized on day 5[5] has a place in final interpretations.

The present findings support the earlier evidence that testosterone propionate may cause skeletal growth changes[1] and body weight increase[6,7] in rats in the absence of the pituitary. This in no way detracts from the importance of the pituitary's role in promoting growth. Rather, it suggests that along with its so-called direct growth hormone

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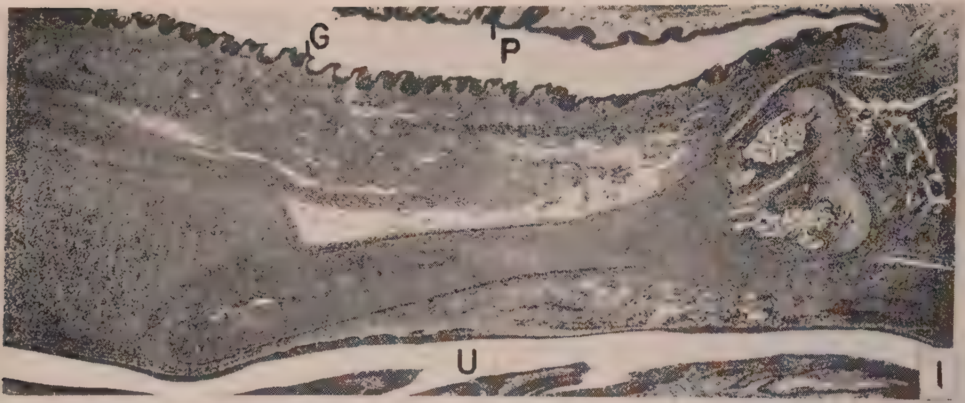
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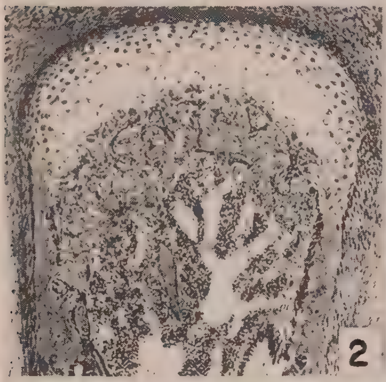
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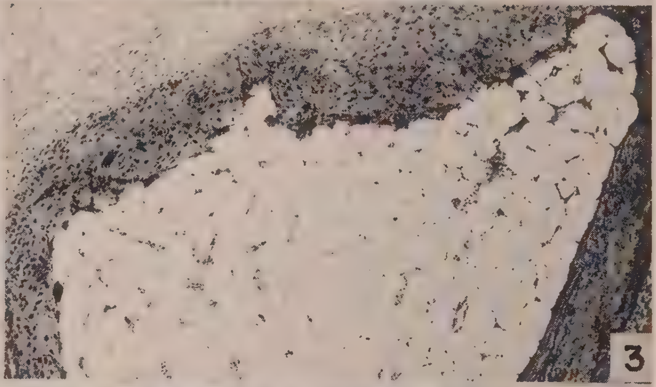
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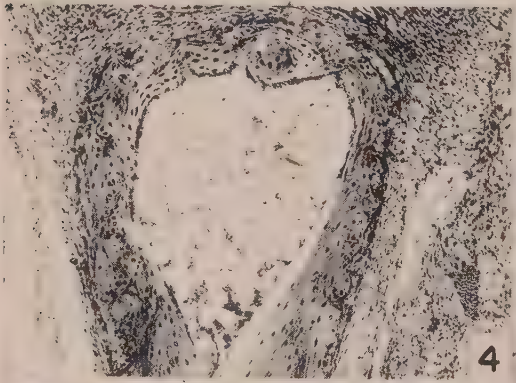
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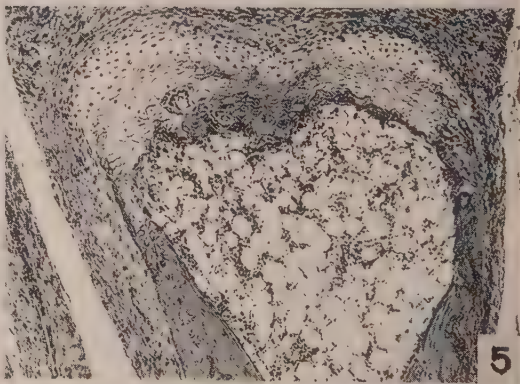
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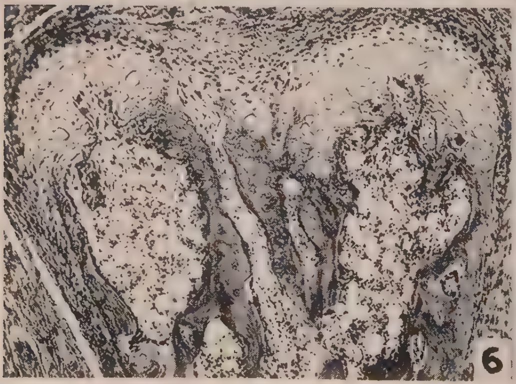
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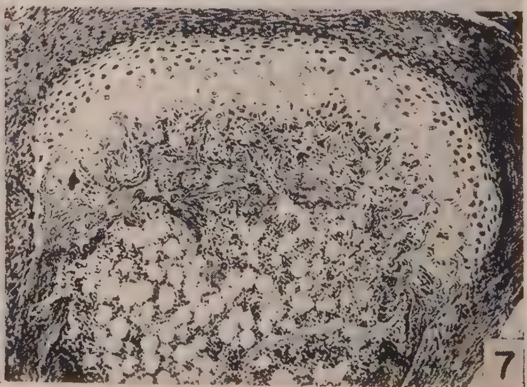
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FIG. 1. The os penis of a rat castrated and hypophysectomized on day 26 and injected, subcutaneously, daily, thereafter for 21 days with 0.1 mg testosterone propionate in sesame oil. Necropsy one day after the last injection. Condyles have developed proximally. The completely ossified distal tip is in close contact with the urethra of the glans and is inserted against a cushion of dense connective tissue and fibro-cartilage which later forms a secondary ossicle. The marrow cavity is inconspicuous except in the condylar region. The balano-preputial epithelial cord has split under the influence of the androgen to form a horny, papillated covering for the glans (G) and the smooth lining of the prepuce (P). See Fig. 6 for higher magnification of the proximal end of the os. H. & E. × 25.

FIGS. 2-7. All H. & E. × 90. Proximal end os penis.

FIG. 2. From an onset control rat 26 days old. Chondrocytes are being formed from the chondrogenic tissue (above) and are being destroyed and replaced by trabeculae of endochondral bone. Periosteal bone is also being formed here and was observed throughout the remaining length of the os. The marrow contained but few fat cells.

FIG. 3. From a 9-month-old, unoperated, untreated, normal rat. Apparently the definitive pattern of this bone has been attained. Although no cartilage cells were recognized the possibility that some of the mesenchyme covering the bone might become chondrogenic remains. The marrow is almost entirely fatty.

FIG. 4. From a 47-day-old rat, hypophysectomized and castrated on day 26 and injected only with sesame oil as a control. Cartilage has disappeared and osteoblasts line communications between the marrow cavity and what was formerly perichondrium (above). Most of the marrow is composed of fat cells. Some of the rats in this group showed a few remaining cartilage cells in this region of the os.

FIG. 5. From a 47-day-old rat, hypophysectomized and castrated on day 26 and injected daily immediately thereafter with 0.01 mg of testosterone propionate. Cartilage has been maintained and has continued to proliferate and to be replaced by bone.

FIG. 6. Same, except for hormone dosage which was 0.1 mg. The marrow in Figs. 5 and 6 has been restored to a condition similar to that of a normal rat of this age (see Fig. 7).

FIG. 7. From a normal, untreated, 47-day-old rat.

(somatotrophin) effect and its indirect action upon skeletal tissues through thyrotrophin, one must also consider the indirect stimulus of its gonadotrophic substances responsible for the elaboration of androgen. Both male hormone and somatotrophin have been shown to perform nitrogen sparing (protein-anabolic) functions[7,8] and Rupp and Paschkis have recently reminded us that thyroid hormone at optimal levels is also a protein anabolizer.[9]

Summary. Rats hypophysectomized and castrated on day 26 and injected immediately thereafter with 0.1 mg testosterone propionate daily for 21 days showed an average daily weight gain of 1 g. During the same period unoperated, uninjected littermate controls gained approximately 5 g daily. The distal segment of the penis of these experimental animals weighed on an average 25% more than the normal 47-day-old controls. The ossicle in this portion of the penis of the experimental group measured 3.80 ± 0.12 mm as contrasted with the normal length of 4.1 ± 0.2 mm and the original length of 3.33 ± 0.15 mm for the 26-day-old normal and the length of 3.20 ± 0.18 mm for the doubly-operated 47-day-old controls uninjected with hormone.

Addendum. Our attention has been called to similar findings reported by Sindram, I. S., *et al.* in *Acta Need. Morph.*, 1939, **2**, 236.

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[8] Kochakian, C. D., Conference on Metabolic Aspects of Convalescence, N. Y., 1946, p. 169.

[9] Rupp, J., and Paschkis, K. E., Abstract 58, 1948 Meeting of the Assoc. for the Study of Internal Secretions.

16652

Effect of Amino Acid Deficiencies on Incorporation of Radioactive Carbon-Labeled Amino Acids into Animal Tissue Proteins.*

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Comparatively little is known of the effect of malnutrition on the metabolic processes of the organism. Accumulation of such information is of importance for the advancement of biochemistry. In the present investigation it is shown by means of isotopic tracer experiments that deficiency of either of the essential amino acid tryptophan or phenylalanine results in impaired ability of the mammalian organism to incorporate C^{14} from labeled amino acids into protein.

The introduction of protein-hydrolysates in therapy has led to the development of the concept that all essential amino acids must be present simultaneously for protein synthesis and growth. The injection of tryptophan a few hours after the injection of a casein-hydrolysate containing no tryptophan failed to induce positive nitrogen balance, while simultaneous administration of the two was successful.[1] Melnick and coworkers[2] attributed the lower biological value of soy protein compared to that of heat-processed soy protein, to a delayed liberation of methionine during digestion in the gastrointestinal tract. When an incomplete mixture of amino acids was fed to rats and the missing essential amino acid supplemented after an interval of several hours, the animals did not grow.[3] It was of interest, therefore, to study the behavior of a tracer dose of a labeled amino acid in an essential amino acid deficiency to obtain information on the effect of the malnutrition on the C^{14} uptake by the tissue proteins of the organism.

Young male rats (Long Evans strain) were placed on a tryptophan deficient diet.[4] Another litter-mate group received the same diet with 0.5% L-tryptophan added. After about 8 weeks, when the symptoms of deficiency had developed, DL-tryptophan-β-C^{14}‡ (0.85 μ C per mg or 105,000 counts per minute per mg) was injected intraperitoneally (3 mg per 100 g body weight). The animals were then fasted for 8 hours and sacrificed. Protein samples from different tissues were prepared for radioactivity assay by homogenizing the tissues and precipitating and washing the protein with trichloracetic acid followed by washings with hot alcohol, alcohol-ether mixture and ether. The radioactivity counts were corrected for self absorption from a curve prepared by counting a highly radioactive sample of protein in layers of different thickness.

In another series of experiments, phenylalanine deficiency was produced in male mice on a synthetic diet,[5] the control group receiving in addition 1.2% DL-phenylalanine. After 3 to 4 weeks, one set of mice received intraperitoneally (3 mg per 100 g body weight) carboxyl-labelled glycine (5.37μc/mg) and another set received labeled tryptophan (2 mg/100 g). The animals were killed 8 hours after injection as in the previous experiment, but in this case they had free access to food. The proteins of the tissues were treated as before.

It is seen from the data (Table I) that the incorporation of the label into the tissue proteins is reduced in essential amino acid de-

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* Aided by grants from the Rockefeller Foundation and the Research Funds of the Medical School.

† Indian Government scholar.

[1] Elman, R., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 484.

[2] Melnick, D., Oser, B. L., and Weiss, S., *Science*, 1946, **103**, 326.

[3] Geiger, E., *J. Nutr.*, 1947, **34**, 97.

[4] Berg, C. P., and Rose, W. C., *J. Biol. Chem.*, 1929, **82**, 479.

‡ Synthesized by Dr. Charles Heidelberger and supplied through the generosity of Dr. Heidelberger and Dr. Melvin Calvin.

[5] Womack, M., and Rose, W. C., *J. Biol. Chem.*, 1947, **171**, 37.

TABLE I.
Activity of C^{14} in Tissue Proteins in Counts per mg per Minute.*

	Tryptophan deficiency		Phenylalanine deficiency			
	C^{14}-Tryptophan administered		C^{14}-Tryptophan administered		C^{14}-Glycine administered	
Organ	Supplemented	Deficient	Supplemented	Deficient	Supplemented	Deficient
Intestines	64.8	28.9	43.9	38.8	197	174
Plasma	52.0	28.0				
Liver	41.5	27.9	20.3	17.7	140	105
Kidney	34.9	21.4	29.7	20.6	118	68.0
Spleen	22.7	9.6	28.2	17.5	114	90.2
Testes	19.6	5.4				
Stomach	30.3	8.0				
Heart	10.5	4.3	8.4	5.1	43.0	29.4
Brain	10.2	4.3				
Skeletal muscle	4.7	2.7	4.5	1.8	23.4	5.0
Red cells	5.0	2.0				

* The data represents the average value of 2 animals in each group.

ficiency both when the animals are starved or fed. The decrease may be due to a decrease in net synthesis of protein or to a lowered rate of turnover, or to both combined. The deficiency of the amino acid could directly limit the quantity of enzymes responsible for the incorporation of the label or the effect may be an adaptive response.

Summary: Animals reared on a diet deficient in an essential amino acid (tryptophan, phenylalanine) showed a reduced incorporation of tracer amounts of C^{14} from labeled amino acids in the tissue proteins.

16653

Passive Transfer of Local Cutaneous Hypersensitivity to Tuberculin.

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Many attempts have been made in the past to transfer tuberculin hypersensitivity passively to normal animals. Most of these attempts have been unsuccessful, and the positive results claimed by some authors have either been seriously contested by others, or have been too irregular to be quite convincing.[1,2] A method which insures a readily reproducible transfer of tuberculin hypersensitivity was first described by Chase[3]; this method had been previously devised by Landsteiner and Chase[4] for the passive transfer of the "delayed type" of skin sensitivity to simple chemical compounds. Briefly, it consists in injecting a suspension of living exudate, spleen or lymph node cells from actively sensitized guinea pigs into the peritoneal cavity or the blood stream of normal animals, following which the latter develop general cutaneous hypersensitiveness to tuberculin within 1 to 3 days. Using the same or slightly modified methods, Kirchheimer and Weiser,[5] Cummings, Hoyt, and Gottshall,[6]

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[1] Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, **41**, 159.

[2] Rich, A. R., The Pathogenesis of Tuberculosis, p. 398, 2nd printing, Springfield, Charles C. Thomas, 1946.

[3] Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 134.

[4] Landsteiner, K., and Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 688.

[5] Kirchheimer, W. F., and Weiser, R. S., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 166.

and Stavitsky[7] have obtained similar results. The object of the present study was to determine whether passive tuberculin hypersensitivity can also be induced locally by the intracutaneous injection of exudate cells from tuberculin sensitive animals.*

The methods were based on those used by Chase,[3] and adapted to the special purpose of this work outlined above. Guinea pigs weighing from 550 to 950 g were used as cell donors. They were infected with a recently isolated virulent human strain of *M. tuberculosis,* by the subcutaneous injection of 0.2 mg (moist weight) of bacilli in saline. 3½ to 7 weeks later the animals were skin-tested with O.T. before each experiment, and those showing the strongest reactions were selected for the production of peritoneal exudate. Each experiment comprised 2 tuberculous and 2 normal cell donors. All 4 animals received 30 cc of paraffin oil intraperitoneally. Forty-eight hours later they were killed, and the peritoneal cavities were washed out with heparinized Tyrode solution. The "tuberculous" and the "normal" exudate cells were recovered from the washings separately by mild centrifugation and washed three times in fresh Tyrode. Finally they were made up with heparinized Tyrode into very thick suspensions, similar in appearance and consistency to slightly diluted pus. 0.1 cc of "tuberculous" and of "normal cell" suspension was then injected intracutaneously into each flank of a young albino guinea pig, 3, 4 or 5 such cell recipients (between 250 and 400 g in weight) being used in each experiment. A few drops of the cell suspension left in the injection syringe were filled into a short capillary tube, which was then centrifuged at 2,500 r.p.m. for ½ hour. In this tube the cell volume/suspension volume ratio could be accurately determined; it amounted to about 2/5, and as it remained constant on further sharp centrifugation, it was adopted as a reliable basis on which to calculate the cell volume actually injected. Thus each cutaneous bleb contained 0.035-0.045 cc of cells, the difference in volume between "tuberculous" and "normal" cells in any particular experiment never being more than 0.005 cc. The cells consisted of 57-75% large mononuclear cells, 8-36% lymphocytes, and 6-22% polymorphonuclear leucocytes, the "tuberculous" cells usually comprising more lymphocytes and less polymorphonuclears than the "normal" cells.

The injection of cell suspension elicited a marked reaction in the skin of the recipients; this consisted, 48 hours after the injection, in a moderately indurated circular area of erythema measuring between 5 and 9 mm in diameter, with a small yellowish center, rarely wider than 1 mm. At this time the recipients were tested with tuberculin. For this purpose two technics were employed: a) the direct injection into the prepared skin sites of 0.1 cc O.T. 1/10; and b) the injection at a distant point (intraperitoneally or subcutaneously) of 1.0-1.5 cc O.T., diluted in an equal volume of saline. The first method was not quite satisfactory, as the control sites (prepared with "normal" cells) reacted to the direct introduction of tuberculin with a slight increase in swelling and redness. This was entirely avoided with the second method ("Fernauslösung"), which was adopted for most of the experiments. The intraperitoneal injection of O.T., however, in the amounts mentioned above, resulted in every case in a fairly severe (but never lethal) anaphylactoid shock. On the other hand, the subcutaneous injection of 1.5 cc of undiluted O.T. was unsatisfactory, owing to protracted absorption of the depot, which remained visible through the abdominal skin for several days. It was felt that the severity of shock was somewhat reduced by the following combination of both routes of administration: 0.25 cc O.T. + 0.25

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[6] Cummings, M. M., Hoyt, M., and Gottshall, R. Y., *Pub. Health Rep.*, 1947, **62**, 994.

[7] Stavitsky, A. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 225.

* H. J. Corper and R. E. Stoner have also attempted to induce passive local hypersensitiveness to tuberculin in guinea pigs by the intracutaneous injection of a mixture of tuberculin and citrated whole blood from actively sensitized animals (*Amer. Rev. Tub.*, 1946, **54**, 305). Their uniformly negative results were probably due to the low proportion of white cells in whole blood, the relatively high dilution of tuberculin used, and the simultaneous injection of both reagents.

cc saline subcutaneously, and 15 minutes later 1.25 cc O.T. + 1.25 cc saline intraperitoneally. Most of the "Fernauslösung" experiments were carried out in this manner.

The results of both types of experiment were clear-cut. The reactions at the skin sites prepared with "tuberculous" cells were well developed 16 hours after tuberculin testing and usually at their height at 24 hours. At this time they presented the aspect of rather small but characteristic tuberculin reactions: an indurated, blanched or livid area (4-9 mm in diameter), surrounded by a diffuse zone of erythema significantly wider than before the injection of tuberculin (10-15 mm). In several instances the center was hemorrhagic, thus completing the picture to that of a typical "cocarde" reaction. Strongly reacting sites became necrotic after 48 to 72 hours, and sloughed off in the next few days. It should be noted that all the reactions were of the delayed type; occasionally very slight blanching of a sensitized site was seen as early as 7 hours after the O.T. injection, but never any earlier. In contrast, no change whatever was observed in the control sites after the introduction of tuberculin at a remote point, and, as has already been mentioned, only a slight increase in inflammatory symptoms was seen after local tuberculin testing.

By way of illustration in one experiment 4 young albino guinea pigs received 0.039 cc "tuberculous" cells intracutaneously in the right flank, and 0.042 cc "normal" cells in the left. The sequence of reactions in one of these animals was as follows:

Forty-eight hours after the cell injections: in both flanks areas of swelling and redness (6x6 mm) with a small yellowish center (1x1 mm). 0.25 cc O.T. + 0.25 cc saline are injected under the abdominal skin, followed 15 minutes later by 1.25 cc O.T. + 1.25 cc saline i.p. Immediate shock of moderate intensity; recovery complete in a few minutes. Twenty-four hours later the inflamed area in the left flank is unchanged. Reaction in the right flank: 8x9 mm white indurated area, sharply demarcated, with 4x5 mm violet-red (hemorrhagic) center; outer zone 10x12 mm angry red diffuse margin. Forty-eight hours after tuberculin: 6x8 mm black necrotic center, 10x12 mm red outer zone; the former sloughed off in a few days.

Five experiments of the kind described above were performed, involving a total of 41 animals, 21 of which were used as cell recipients. In 15 of these, transfer of tuberculin hypersensitivity was successful, as evidenced by medium to strong tuberculin reactions at the specifically prepared skin sites. In one experiment an attempt was made to determine whether local sensitivity is preceded by a latent period: the 5 recipients used, including one animal tested 48 hours after the cell injections, gave negative or at best only suggestive reactions, and had to be recorded as failures. In another experiment the duration of local reactivity was explored; this was found to last up to 5 days, and the one recipient in the experiment which was tested 7 days after sensitization failed to react at all. Tests carried out with control glycerine broth gave entirely negative results, whether the broth was injected locally into prepared skin sites or intraperitoneally (when it also caused anaphylactoid shock).

Conclusions. The method of local passive sensitization so extensively employed in human allergy (Prausnitz-Küstner reaction) and in experimental anaphylaxis (Opie and others), can also be applied to the cellular transfer of tuberculin hypersensitivity discovered by Chase.[3] Compared with the latter's experimental procedure, this method offers the advantage of considerably reducing the number of cell donors necessary for successful transfer. The technic used is, however, susceptible of improvement, as in the presence of a marked primary reaction of cavy skin to the injection of homologous exudate cells none but fairly strong tuberculin reactions can be recorded with confidence.

16654

Action of Acetylcholine, Histamine and Cholinesterase Inhibitors on Isolated Ileum in Relation to its Survival Time.*

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In preliminary experiments it was found that the atropinized ileum of the guinea pig began to react to acetylcholine 2½ hours after the death of the animal. Observations of this nature were made by Le Heux,[1] who showed that the rhythmic peristaltic contractions of the isolated guinea pig ileum could no longer be blocked by atropine after the intestine had been kept in Locke solution for several hours; he also noticed a similar behavior in the isolated small intestine of the rabbit. We therefore decided to study the correlation between the time elapsed after death and the responses to acetylcholine, histamine and various cholinesterase inhibitors of the atropinized and non-atropinized intestinal strips of several species of animals.

Experimental. Guinea pigs, rabbits, rats and chickens were used. Strips of ileum were suspended in a Tyrode bath of 45 ml at 37°C. The temperature of the bath for the chicken intestine was kept at 41°C. The experiments were started within 15 minutes after death.

At the beginning of each experiment, several successive doses of 5 to 10 μg acetylcholine bromide were added to the bath. At the height of the second contraction caused by acetylcholine (one minute after addition of the drug), histamine phosphate was added to the bath. After each contraction, the Tyrode solution was changed and a resting period of 3 minutes was allowed.

Acetylcholine bromide and histamine phosphate were added in amounts which caused submaximal contractions. Satisfactory results were obtained by adding the following doses, expressed in μg, to a bath of 45 ml volume:

	Guinea pig	Rat	Rabbit	Chicken
Acetylcholine bromide	0.2-0.5	0.2-0.6	0.2-0.6	0.05-0.3
Histamine phosphate	0.5-1.0	60. -150	10. -40	5. -10

These doses correspond to those recommended by other authors.[2-6]

The cholinesterase inhibitors were used in the following amounts: Physostigmine sulfate: 0.5-10 μg; neostigmine methylsulfate: 1-10 μg; hexaethyl tetraphosphate: 10 μg.

When the intestine was treated with both acetylcholine and histamine, the second drug was added only after the first contraction had reached its maximum. The same precaution was observed when histamine was followed by acetylcholine.

In one series of experiments the intestinal strips were suspended in atropinized Tyrode solution. The intestine was considered to be completely atropinized when it failed to respond to repeated additions of 10-50 μg acetylcholine bromide. For this purpose the following concentrations of atropine were found to be satisfactory: 1 mg atropine sulfate per liter Tyrode for the guinea pig, 2 mg per liter for the rat and 3 mg per liter for the chicken and rabbit. Histamine was used in the same amounts as in the case of non-atropinized intestines.

In all experiments control strips from the same animal were kept in regular and atropinized Tyrode solution for several hours at room temperature or in a bath at 37°C. When the piece of ileum which was subjected to frequent stimulation began to show marked and reproducible changes in its reaction to histamine, it was replaced by a control strip. Then, by registering the contractions, it was observed whether the control strip, which had not been stimulated before, showed the same types of response.

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* This work was aided by a grant from the Office of Naval Research N6ori-20, Task Order 11.

[1] Le Heux, J. W., *Arch. f. d. ges. Physiol.*, 1920, **179**, 177.

[2] Levy, J., and Michel, Z., *J. Physiol. et Pharm. gén.*, 1937, **35**, 389.

[3] Kahlson, G., *Arch. f. exp. Path. u. Pharmakol.*, 1934, **175**, 189.

[4] Page, I. H., and Schmide, E., *Z. f. Physiol. Chem.*, 1930, **191**, 262.

[5] Ambache, N., *J. Physiol.*, 1946, **104**, 266.

[6] Erspamer, V., *Arch. f. exp. Path. u. Pharmakol.*, 1940, **196**, 343.

Results. In the guinea pig ileum, in non-atropinized media, the contractions caused by acetylcholine and by histamine mutually reinforced each other during the first hour after death. (Fig. 1, 2). Toward the end of the third hour when histamine was added to the bath, after the intestine had been contracted by acetylcholine, there was a small initial contraction immediately followed by a profound relaxation. The muscle then recovered and gradually reached the level of contraction attained prior to the addition of histamine. (Fig. 3.) The situation was different when, at the height of a histamine contraction, acetylcholine was added to the bath. Acetylcholine always reinforced the histamine contraction. At the beginning of the experiment simple summation occurred. However, toward the end of the third hour, the contraction initiated by the addition of acetylcholine was interrupted by a series of large peristaltic waves. These in-

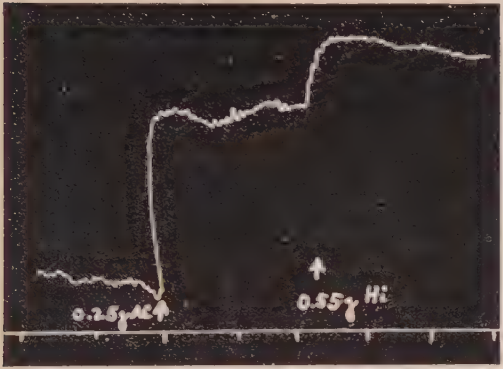

FIG. 1.
Effect of acetylcholine and histamine on the isolated guinea pig ileum ½ hour after death of the animal. Consecutive contractions are additive. Time in 30 second intervals.

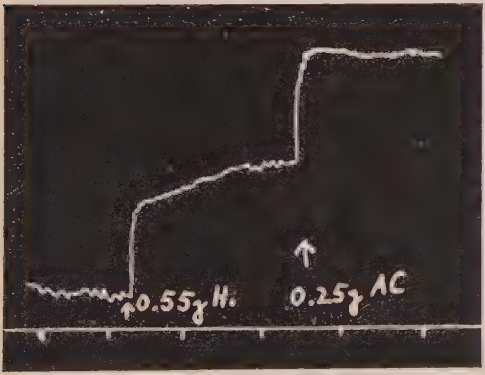

FIG. 2.
Effect of histamine and acetylcholine on the isolated guinea pig ileum ½ hour after death of the animal. Time in 30 second intervals.

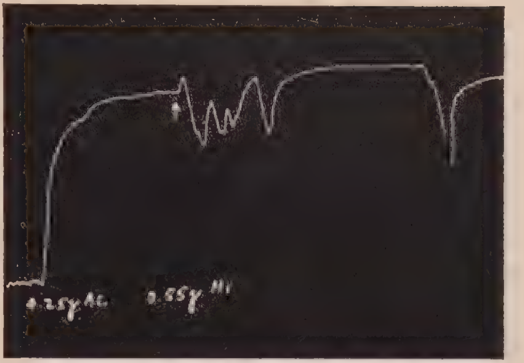

FIG. 3.
Effect of acetylcholine and histamine on the isolated guinea pig ileum 3½ hours after death of the animal. Histamine causes relaxation when added after acetylcholine.

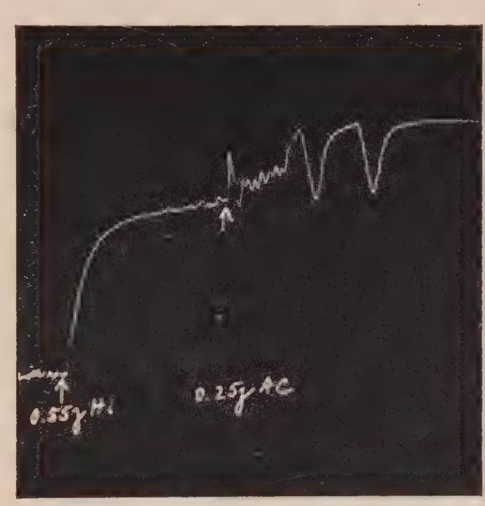

FIG. 4.
Effect of histamine and acetylcholine on the isolated guinea pig ileum 3½ hours after death of the animal. Acetylcholine reinforces the contraction caused by histamine.

TABLE I.
Sensitivity of the Isolated Guinea Pig Ileum to Acetylcholine at Different Intervals After Death.

Time after death in min.	Acetylcholine to 45 ml bath fluid μg	Height of contraction in mm
10	10	42
15	10	72
20	10	96
25	10	96
30	10	87
At 35 min. atropinized Tyrode was added (1 mg atropine sulfate/liter)		
40	10	0
90	10	0
120	10	0
135	10	0
140	10	3
150	10	9
180	10	18
195	10	15

creased in magnitude and frequency as the experiment proceeded. Eventually the muscle reached a plateau which was invariably higher than the one which it maintained after the first addition of histamine. (Fig. 4) Control muscles kept in Tyrode solution for several hours before the addition of histamine reacted in the same way as those which had undergone frequent stimulation.

In the rabbit ileum histamine and acetylcholine mutually reinforced each other during the first 2 hours after death. From the third or fourth hour on, a summation occurred only when histamine was followed by acetylcholine, but not when acetylcholine was followed by histamine.

During the first hour after death histamine elicited contraction of the rat intestine. As early as 2 hours after death the same dose of histamine caused relaxation or provoked no response. In the chicken intestine, 3 hours after death, the reaction to histamine became reversed at a time when the intestine was still responding with the usual contraction to the addition of acetylcholine.

The contractions caused by the cholinesterase inhibitors had the following characteristics: 1. The response was delayed while the response to histamine was always immediate. The delay between the addition of the drug and the contraction was most marked in the guinea pig. In all 4 species the time interval between the addition of the drug and the contraction was longest with physostigmine, least pronounced with neostigmine. 2. The response was gradual, *i.e.,* it took several minutes before the muscle reached the height of its contraction. Within the range of doses used, the delay in the response and the amplitude of the contraction were largely independent of the dose administered. 3. The intestinal strips still reacted to acetylcholine and to the cholinesterase inhibitors with a contraction at a time when histamine provoked no more response or relaxation only.

A total of 12 experiments was carried out on rabbits, 20 on rats and 9 on chickens. Of these 9 rabbits, 18 rats and 6 chickens showed the above described phenomena. Without exception, the small intestines of the individuals which did not undergo these changes responded with unusually sluggish contractions both to acetylcholine and to histamine. In some instances such delayed responses could be ascribed to the fact that the animals were old, or that the intestines had been subjected to prolonged handling before stimulation. However, in the case of the guinea pig, in all 24 animals which were used during the winter months, the reaction of the intestine underwent the changes described above. In 8 experiments which were carried out in the spring on the same strain of guinea pigs, the intestine reacted in a sluggish way to acetylcholine and to histamine. A decreased sensitivity of the isolated guinea pig ileum to histamine in the springtime has been reported by Kwiatkowski.[7]

In all experiments, the atropinized ileum of all 4 species did not react to acetylcholine and to the cholinesterase inhibitors during the first 2 to 3 hours after death. At the time when the non-atropinized intestine began to fail to contract from histamine, the responses of the atropinized muscles to acetylcholine and to cholinesterase inhibitors were no longer blocked by atropine in the same concentration which was sufficient to inhibit the action of acetylcholine immediately after death. A typical experiment is shown in Table I.

Discussion. From our experiments it seems that several hours after death a certain change occurs in the intestinal muscle. This change in the surviving tissue results in a reversal of the response to histamine at a time when the muscle still contracts to acetylcholine and to the cholinesterase inhibitors. These observations do not support the recent theory[5] that histamine contracts the smooth muscle by causing a liberation of acetylcholine. The different character of the contractions caused by histamine and by the cholinesterase inhibitors and the different reactions to atropine are also against the theory that histamine stimulates the smooth muscle of the intestine through a cholinergic mechanism. On the other hand, the possibility cannot be excluded that there is "an interdependence between the sites of attack" of histamine and acetylcholine as was suggested by Rocha e Silva.[8] It is also obvious that when experiments are carried out with isolated intestinal muscle, it is necessary to take into account the time which has elapsed since the death of the animal, particularly when histaminic responses and the reactions of the atropinized muscles to parasympathetic stimulants are being observed.

Summary. The action of acetylcholine, histamine, physostigmine, neostigmine, and hexaethyltetraphosphate was studied on the isolated ileum of the guinea pig, rabbit, rat and chicken in non-atropinized and atropinized media. In all 4 species the contractions caused by acetylcholine and histamine reinforced each other immediately after the death of the animal, regardless of the order in which they were added to the bath. Two and a half hours to 3 hours after death, histamine failed to contract the intestine when it was added at the height of an acetylcholine contraction. In the rat, rabbit and chicken the intestine no longer responded to histamine at a time when it still reacted to acetylcholine and to the cholinesterase inhibitors. In all 4 species atropine failed to block the action of acetylcholine and cholinesterase inhibitors in intestinal strips which were at least 3 hours old.

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[7] Kwiatkowski, H., *J. Physiol.*, 1943, **102**, 32.

[8] Rocha e Silva, M., *J. Pharm. Exp. Ther.*, 1944, **80**, 399.

16655

Renal Plasma Flow During Moderate Exercise of Several Hours' Duration in Normal Male Subjects.

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That exercise produces a decline in renal plasma flow is now established.[1,2] In a previous report[2] the degree of reduction was found to be progressive for at least 30 minutes as exercise proceeds and was directly related to the severity of the exercise. The effects of long-sustained exercise have not, to our knowledge, been determined and it is with this topic that the present report deals.

Method. Technically the method employed in this work was identical with that previously described[2] except that the time spent on the treadmill totalled 3 hours. Para-amino-hippurate was used to determine renal plasma flow. As in the previous experiments a moderate water diuresis was instituted and urine samples were collected by voluntary micturition. The exercise was at the rate of 3 miles per hour at 5% grade.

Material. Four normal male students, aged 22 to 27 years, were employed for the study. Three of the subjects had been studied extensively in previous experiments on the effects of exercise of relatively short duration. A single experiment employing exercise of long duration was done on each subject.

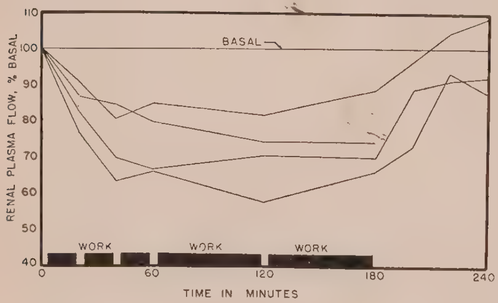

Fig. 1.

Changes in renal plasma flow in 4 normal male subjects during and after walking 3 m.p.h. at 5% grade.

Results. Fig. 1 and Table I summarize the effects of long-continued exercise on the renal plasma flow in these 4 subjects. At this level of exercise the renal plasma flow declined to a relatively stable value during the first 40 to 60 minutes of exercise. The value reached during the first hour was relatively well maintained in these 4 subjects for the remaining 2 hours of exercise. The degree of decline in the first 32 minutes of work corresponded almost exactly to that determined in previous experiments[2] at the same level of work.

In 2 of the subjects the renal plasma flow had returned to within 10 per cent of resting levels 40 minutes after cessation of work. In a third subject the resting value was slightly exceeded at the end of 40 minutes. No studies of recovery were carried out on the fourth subject.

Discussion. The decrease in renal plasma flow in response to moderate exercise is, it appears, a progressive phenomenon only during the first 40 to 60 minutes of the exercise. Continuation of the stress for 2 full hours beyond this point resulted in no further decline in the amount of plasma flowing through the kidney. It is well established that the cardiovascular system can adjust relatively quickly to various levels of work, permitting the work to be continued without further circulatory adjustment until the subject approaches the point of exhaustion. That the kidney participates in the circulatory changes is clearly shown in this and previous experiments. The shunting of blood away from the kidney reaches a maximum relatively early in sustained exercise and is continued until the work ceases. The adjustment of renal plasma flow in response to exercise is, however, not as rapid as some other circulatory changes. In

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[1] Barclay, J., Cooke, W., Kennedy, R., and Nutt, M., *J. Physiol.*, 1945-46, **104**, 14P.

[2] Chapman, C., Henschel, A., Minckler, J., Forsgren, A., and Keys, A., *J. Clin Invest.*, 1948, **27**, 639.

TABLE I.
Renal Plasma Flow in cc per Minute, Corrected to 1.73 sq. m. Body Surface, During and After Work of Relatively Long Duration at the Rate of 3 m.h.p. at 5% Grade.

		Work					Recovery		
Subject	Basal	20 min.	40 min.	1 hr	2 hr	3 hr	20 min.	40 min.	60 min.
BS	512	416	315	332	360	355	452	458	471
PW	576	499	487	459	425	425	—	—	—
WW	523	484	431	452	434	462	—	557	723
SN	607	466	385	402	345	399	456	566	535
Mean	555	466	405	411	391	410	454	516	578

work of this type, the increase in pulse rate and oxygen uptake, for example, are maximal 10 to 15 minutes after initiation of exercise.

Presumably, as the subject approaches exhaustion other changes in the renal plasma flow take place but in none of our experiments were the subjects seriously taxed by the work imposed.

Recovery was studied in 3 subjects for one hour after cessation of exercise. Although the number of experiments is too small to permit statistical testing, the speed of the recovery process after 3 hours of exercise seems to be much the same as after only 32 minutes of this work. In neither case was it as rapid as return of the pulse rate or blood pressure to resting levels.

Summary. 1. Renal plasma flow was studied by the para-amino-hippurate clearance method during moderate exercise of relatively long duration in 4 healthy young men. 2. During the first 16 minutes of walking 3 m.p.h. at 5% grade the renal plasma flow fell from 9 to 23.2% (of the resting control); during the second 16 minute period it fell from 15.4 to 36.6% and during the third 16 minute period from 18.5 to 33.7%. 3. During the second and third hours of exercise (2 consecutive 56-minute periods), there was little further change in the renal plasma flow. 4. In 2 of 3 experiments where recovery was studied, the renal plasma flow had returned to within 10% of the resting control value one hour after cessation of exercise.

16656 P

Asymmetrical Suppression of Vertebral Epiphyseal Growth with Ionizing Radiation.

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(Introduced by L. J. Soffer.)

From the Orthopedic and Radiation Therapy Services, Mount Sinai Hospital, New York City.

Ionizing radiation can suppress growth of epiphyses. Many studies of this phenomenon have been made on long bones, but only Engel[1] has previously described the deformities resulting from irradiation of the growing spine. He placed radium needles alongside the spines of young goats and produced a wedge deformity of the adjacent vertebrae. The narrowing of the vertebrae occurred on the side nearer the radium, and the resultant scoliosis was concave on the same side.

We have tried additional technics in radiation of the spine in growing animals. Using radon seeds for interstitial radiation and 140 kV x-rays for external radiation, our preliminary experiments have attempted to establish a useful dosage range for suppression of vertebral epiphyseal growth in baby rabbits.

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* Dazian Research Fellow in Radiology.

[1] Engel, D., *Am. J. Roentgenol. and Rad. Therapy*, 1939, **42**, 217.

Interstitial Radiation. Radon seeds were implanted just lateral to a lumbar intervertebral space. Films of the spine were made with seeds *in situ* (Fig. 1) in order to evaluate positioning and dosage. Fourteen rabbits had a total of 24 seeds implanted; 10 rabbits had two seeds in each, and 4 animals had 1 seed in each. These seeds were equivalent to 0.2, 0.4, or 0.6 millicurie of radium on total decay and were filtered by the equivalent of 0.3 mm of platinum. Of these 14 animals 10 died of gastro-intestinal infections less than 3 weeks after implantation of radon seeds. The 4 surviving animals had a total of 6 seeds. Wedging occurred adjacent to 2 seeds, both of which were placed in apposition with the intervertebral space and contained 0.6 mc of radon on total decay. (Fig. 2) Other implanted sites did not show wedging because of poor positioning of seeds or lower radon content of the seeds.

External Radiation. Asymmetrical radiation of the growing epiphyses of the vertebral bodies was also effected by directing a narrow beam of x-rays to one side of the lumbar spine. Under intravenous anesthesia to immobilize the rabbits one side of the spine was treated with dosages (in air) ranging from 300 to 1200r. Radiation factors were: 140 kV, 20 ma., 30 cm target skin distance, filtration of 0.25 mm Cu, and 1 mm Al., HVL 0.50 mm Cu., and a flux of 60 r/min. The field size was 1 x 7 cm, and the dose at the middle† of the vertebral bodies at a depth of 1.5 cm was 70% of the air dose[2,3] at the center of the field. Accuracy of field was verified by exposing x-ray films with the rabbits under anesthesia and with the therapy machine in place ready for treatment (Fig. 3).

Of rabbits irradiated externally 11 died of

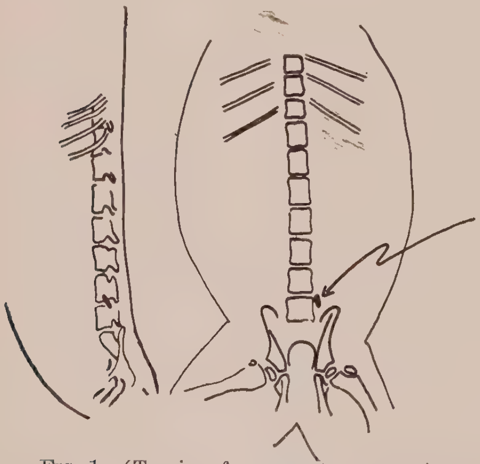

FIG. 1. (Tracing from roentgenogram).
Anteroposterior and lateral roentgenograms made immediately after implantation to show position of implanted radon seed (arrow). The spine is straight.

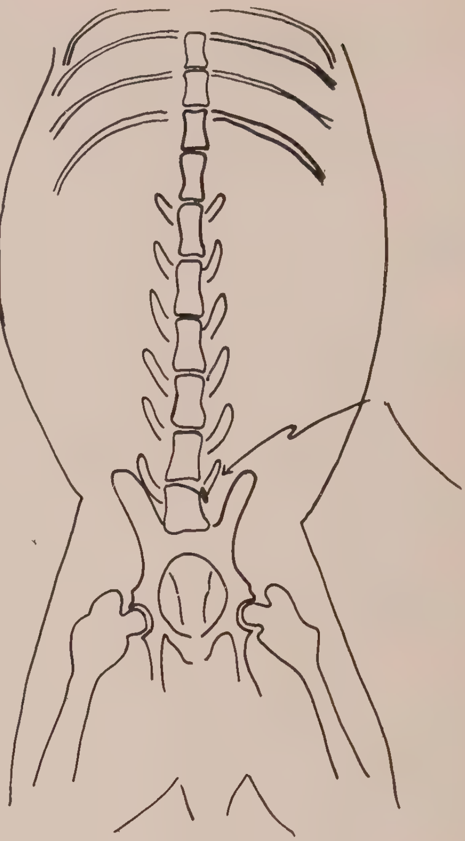

FIG. 2. (Tracing from roentgenogram).
Same rabbit as in Fig. 1. This film was made 110 days after implantation of radon seed and illustrates asymmetrical growth of both vertebrae adjacent to the 0.6 mc. radon seed (arrow). In this instance the middle of the epiphyses nearest the seed received an estimated dose of 2680r while the opposite side of these vertebral epiphyses received 670r. The growth suppression varied directly with the dosage.

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† Midway between anterior and posterior edges.

[2] Glasser, O., Quimby, E. H., Taylor, L. S., and Weatherwax, J. L., Physical Foundations of Radiology, Paul B. Hoeber, Inc., N. Y., 1944.

[3] Clarkson, J. R., *Brit. J. Radiol.*, 1941, **14**, 265.

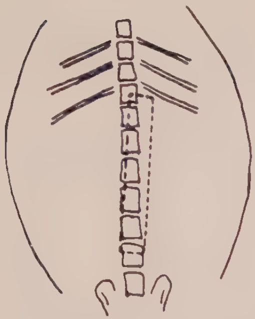

Fig. 3. (Tracing from roentgenogram). This shows the spine of a three week old rabbit with 1 × 7 cm radiation field indicated by dotted line. Dose to the middle of the vertebral bodies involved was 1000r.

gastro-intestinal infections. The five survivors received dosages to the region midway between the anterior and posterior surfaces of the vertebral bodies of 100r, 350r, 700r, or 1000r. Only the 2 animals which received the highest dosage showed marked wedging on the irradiated side of the vertebrae. (Fig. 4). Slight wedging was seen in an animal which received 700r.

Summary. The lumbar spines of 30 young rabbits were unilaterally irradiated with radon seeds and X-rays. Twenty-one animals died of gastro-intestinal disturbances unrelated to radiation. Five of the 9 survivors showed wedging of the vertebral bodies with the narrowing of the bodies on the more heavily irradiated side. Dosage estimates indicate that growth of the epiphyses of the vertebral bodies of baby rabbits may be suppressed with 700 to 1000 r to the epiphysis. Doses of 350 r or less have no apparent effect on the growth.

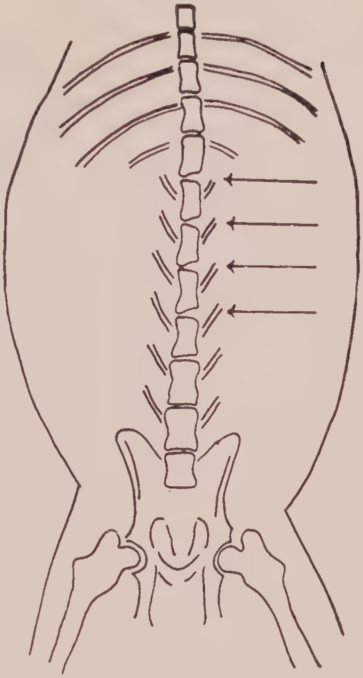

Fig. 4. (Tracing from roentgenogram). This shows the spine of the same rabbit 54 days later. Wedged vertebrae are indicated by arrows.

16657 P

Urinary Excretion of Aureomycin.*

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From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

Aureomycin is a new antibiotic which is active against many gram-positive and gram-negative bacteria and also against certain viral and rickettsial infections.¶ As part of a clinical trial of this antibiotic in a number of infections, bacteriologic and pharmacologic studies were also carried out in this laboratory. The results of the bacteriological studies are reported elsewhere.[1] In this paper are presented some preliminary findings on the urinary excretion of aureomycin after single and multiple doses.

Two normal adult male subjects were given a single dose by mouth in the morning on a fasting stomach; one was given 0.5 g and the other 0.75 g. Each subject emptied his bladder just prior to taking the dose and the urine thus voided was used as a control. The bladder was then emptied at intervals, the volumes of urine recorded and aliquots immediately filtered,** frozen and stored at –20°C. After all the urines had been collected, each specimen was assayed for aureomycin by a method similar to that used by Rammelkamp for penicillin.[2] The test organism, *Streptococcus* No. 98, was completely inhibited by 0.5 μg per ml and partially inhibited, as evidenced by growth on subculture, by 0.25 to 0.125 μg per ml. The control urine specimens, obtained before and several days after the dose, failed to inhibit the growth of the test organism.

The results for the subject receiving 500 mg are shown in Fig. 1 and those for the subject receiving 750, who had a much greater volume of urine output, are shown in Fig. 2. The greatest rate of aureomycin excretion occurred in both instances between 4 and 8 hours. If similar absorption and excretion take place from repeated oral doses, the intervals between such doses should be about 8 hours in order to maintain maximum absorption and excretion rates. Concentrations of aureomycin sufficient to completely inhibit the test organisms when the urine was diluted 16-fold or more were excreted for at least 33 hours. The aureomycin was still being ex-

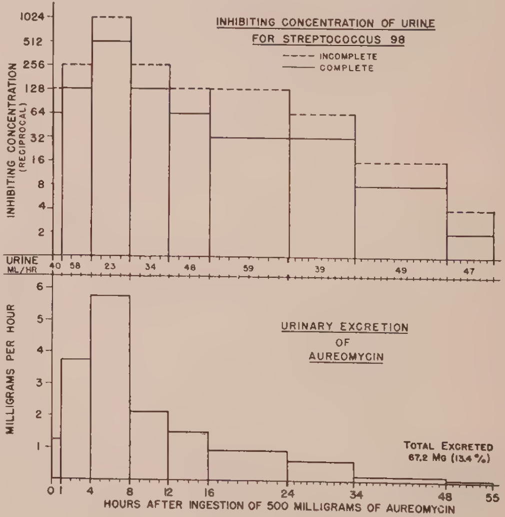

Fig. 1.

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* Aided by a grant from the United States Public Health Service.

† Research Fellow in the Field of Virus Diseases, National Research Council.

‡ Research Fellow, American College of Physicians.

§ Clare Wilcox, Janice M. Bryan, and Paul F. Frank rendered technical assistance.

¶ Reports on the method of isolation of aureomycin, its pharmacology and activity will be made by workers of the Lederle Laboratories Division of the American Cyanamid Company at the New York Academy of Sciences, July 21, 1948. Some of the data were made available by these workers in personal communications to the authors.

[1] Paine, T. F., Jr., Collins, H. S., and Finland, M., *J. Bact.*, 1948, **56**. Submitted for publication.

** Filtration through Seitz, Mandler or sintered glass filters did not remove any of the antibiotic.

[2] Rammelkamp, C. H., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 95.

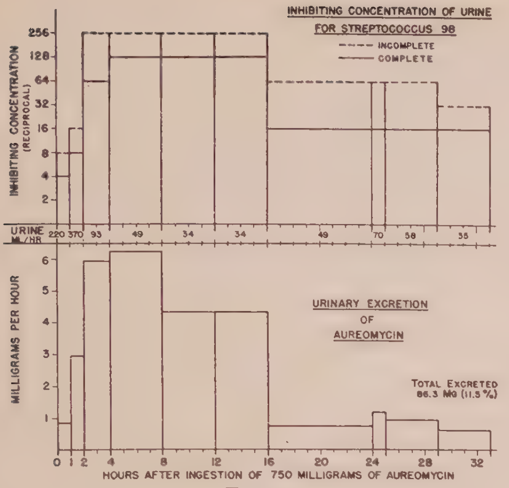

Fig. 2.

creted in both subjects at 55 and 33 hours, respectively, when the studies were terminated. The total amount recovered was 13.4% of the 500 mg dose in 55 hours and 11.5% of the 750 mg dose in 33 hours.

Blood levels carried out with the same organism were unsatisfactory since the maximum amount of antibiotic in the serum inhibited the test organism only in a final dilution of 1:2 or 1:4.

Total urinary excretion studies were made in 2 patients who were receiving 2 doses of aureomycin by mouth each day for several days. The urines were collected at 12-hour intervals and all voidings were stored at 5 to 10°C during the intervals. The tests were otherwise carried out in the same manner as before. In one of these patients who received 0.25 g twice a day for 10 days the concentrations of antibiotic in the urine were sufficient completely to inhibit the test streptococcus in dilutions of 1:4 to 1:16, and partial inhibition occurred in dilutions of 1:8 to 1:32. In the patient who received 0.5 g twice a day for 7 days the concentrations of aureomycin in the urine ranged from 10 to 32 μg per ml. In these 2 patients the total amount recovered was 5% and 2%, respectively of the amount administered. Antibiotic activity was present in the urine up to 72 hours and longer after the last dose. Control urines from these patients, obtained before and several days after the antibiotic was given failed to inhibit the test strain.

Both of the patients were acutely ill and the differences in the percentage recoveries may have been related to poor absorption of the drug associated with the illness. Technical difficulties may also have accounted, in part, for the discrepancies.

Summary. After a single oral dose of 0.5 or 0.75 g of aureomycin given to fasting normal subjects antibiotic activity was recovered in the urine for more than 33 to 55 hours. The antibiotic was excreted in high concentrations between 2 and 16 hours and the largest rate of excretion occurred between 2 and 8 hours. The findings suggest that the optimum intervals between oral doses of aureomycin should be about 8 hours.

16658 P

Stereoencephalotomy.*

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From the Departments of Experimental Neurology and Neurosurgery, Temple University School of Medicine and Hospital, Philadelphia.

Using the previously described stereotaxic apparatus (stereoencephalotome) for the production of subcortical lesions in the human brain,[1] the following procedures have been developed.

I. *Dorsomedial thalamotomy* in mental dis-

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* Aided by a grant from the Am. Med. Assn. Committee on Scientific Research.

[1] Spiegel, E. A., Wycis, H. T., Marks, M., and Lee, A. J., *Science*, 1947, **106**, 349.

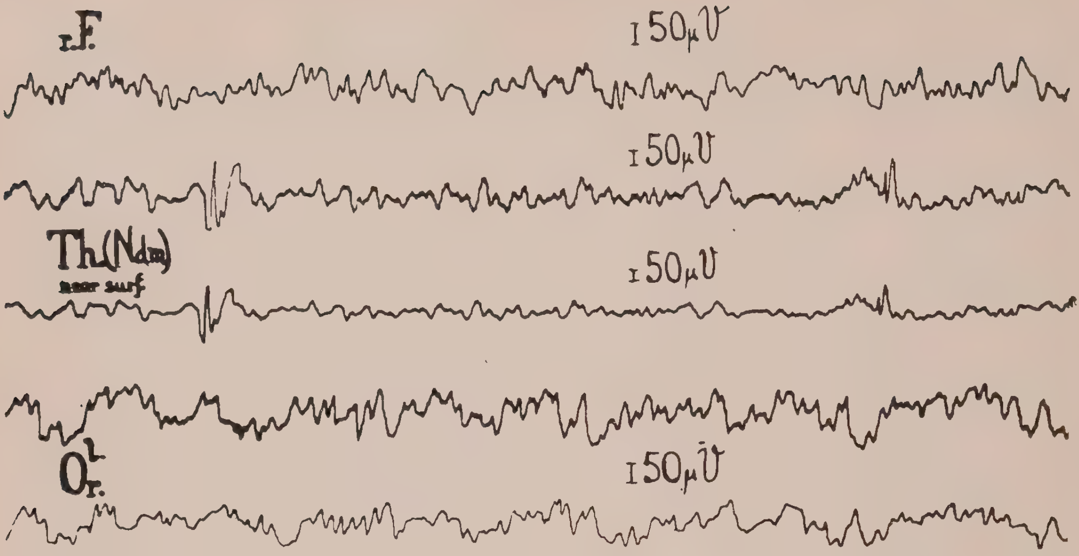

FIG. 1.
Electrothalamogram (Th) recorded from the dorsomedial nucleus near its surface simultaneously with the electroencephalogram recorded from the scalp in the frontal (F) and occipital (O) areas in a patient suffering from grand and petit mal attacks (patient under pentothal-ether-nitrous oxide anesthesia).

orders. Lesions have been placed bilaterally in the dorsomedial nucleus of the thalamus in mental disorders with a prevalent emotional component, in order to interrupt the cortico-diencephalic connections probably related to the mechanism of emotions (16 cases). Lesions covering about one seventh of the volume of this nucleus on each side have been able to produce relief of nervous tension, anxiety, depression, irritability, agitation, hallucinations or compulsions; this has been obtained[2] without at least some of the undesirable by-effects of prefrontal lobotomy such as epileptiform convulsions, childishness, facetiousness, distractibility, lack of tact or discipline.

II. *Mesencephalothalamotomy* (mty) for relief of intractable pain. Lesion of the long ascending pain-conducting pathways in the midbrain has been combined with a lesion of the dorsomedial nucleus. Since patients become unconcerned about thair pain following prefrontal lobotomy (Freeman and Watts[3]), and since the dorsomedial nucleus degenerates following this procedure, a lesion of this nucleus is added to that of the long ascending pain-conducting pathways in order to induce a relative indifference to pain perceived through remaining auxiliary pathways. In a woman suffering from unilateral facial pain for 6 years despite retrogasserian rhizotomy, mty on the opposite side abolished the pain completely except for occasional paresthesias in the maxillary region.[4] In a second patient suffering from diffuse burning pain and spasms in the right lower extremity following an injury to the lumbar spine and unrelieved by sympathectomy, rhizotomy and bilateral chordotomies, the bilateral mty reduced the painful area to the region around the right buttock, while the spasms of the leg and the sensation of the muscular contractions persisted.

III. *Paracommissural thalamotomy* in petit mal. In cases suffering from grand and petit mal attacks, lesions were placed bilaterally at the level of the commissura media around the medial part of the internal lamina medullaris.[5] Electric stimulation of this region in experimental animals yields a spike and dome pattern in the electroencephalogram (Jasper[6]). Preceding this lesion, the electrothalamogram was recorded simultaneously with the electroencephalogram;[7] spike discharges were found in the electrothalamogram.† These spike discharges were not limited to the immediate vicinity of the internal lamina medullaris but sometimes showed the maximum in more dorsal parts of the thalamus close to the ventricle (Fig. 1). In two cases, the operation did not influence the grand mal attacks; it diminished, at least transitorily, the frequency of the petit mal attacks and the severity of accompanying motor phenomena.

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[2] Spiegel, E. A., Wycis, H. T., and Freed, H., Joint Meeting Philadelphia and New York Neurologic. Soc., April 23, 1948.

[3] Freeman, W., and Watts, J. W., *Lancet*, 1946, **1**, 953.

[4] Spiegel, E. A., and Wycis, H. T., *Wien. med. Wochenschr.*, in press; Wycis, H. T., Soloff, L. and Spiegel, E. A., Philadelphia Neurolog. Soc., Feb. 27, 1948.

Summary. The stereotaxic technique has been applied to the human brain for relief of mental disorders with a prevalent emotional component and of intractable pain. In epileptics suffering from grand and petit mal, the electrothalamogram was recorded, revealing spike discharges; lesions placed in the vicinity of the commissura media diminished frequency and severity of the petit mal attacks.

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[5] Spiegel, E. A., Wycis, H. T., Freed, H., and Lee, A. J., Am. Neurol. Assn., June 16, 1948.

[6] Jasper, H. H., and Droogleever-Fortuyn, J., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1947, **26**, 272.

[7] Wycis, H. T., Spiegel, E. A., and Lee, A. J., Intl. League against Epilepsy, Am. Branch, June 13, 1948.

† Gibbs, Hayne and Gibbs[8] also recorded spike discharges from various subcortical areas in petit mal. The authors inserted the recording electrode into the brain of the patients without the guidance of a stereotaxic apparatus, trying to establish the location by x-ray studies after air injections into the ventricles.

[8] Gibbs, F. A., Hayne, R., and Gibbs, E. L., Int. League against Epilepsy, Am. Branch, June 13, 1948.

16659

Possible Role of Free Phenols in Renal Uremia.*

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The work of Becher and his associates,[1-6] has stressed the importance of free phenols in blood as a major factor in the development and intensification of the uremic syndrome. In an extensive review of the pathogenesis of this syndrome, Harrison and Mason[7] state that "whether in patients with nephritis phenol retention is sufficiently marked to produce further renal damage is an interesting but still unsettled problem." Dickes,[8] using the method of Theis and Benedict[9] for determining blood phenols, noted that 21 out of 23 uremic patients showed a direct correlation between the degree of cerebral depression and the height of the blood phenols. However, the correlation of total phenols was

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* Data from thesis submitted in partial fulfillment of the requirements for the Degree Master of Science.

[1] Becher, E., *Deut. Arch. f. Klin. Med.*, 1925, **145**, 333.

[2] Becher, E., *Ergeb. d. ges. Med.*, 1933, **18**, 51.

[3] Becher, E., and Koch, F., *Deut. Arch. f. Klin. Med.*, 1925, **148**, 78.

[4] Becher, E., and Litzner, S., *Klin. Wochschr.*, 1926, **5**, 147.

[5] Becher, E., and Litzner, S., *Klin. Wochschr.*, 1926, **5**, 1373. Cited *Chem. Ab.*, 1926, **20**, 3192.

[6] Becher, E., Litzner, S., and Taglich, W., *Z. f. Klin. Med.*, 1926, **104**, 182.

[7] Harrison, T. R., and Mason, M., *Medicine*, 1937, **16**, 1.

[8] Dickes, R., *Arch. Int. Med.*, 1942, **21**, 662.

[9] Theis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1924, **61**, 67.

TABLE I.
Blood Phenol Determinations—Method of Schmidt.
Diagnosis: Uremia of Renal Origin.

Case No.	N.P.N. mg %	Free phenol mg %	Conjugated phenol mg %	Total phenol (Free plus conjugated) mg %
1	105	.050	.519	.569
2	210	.164	—	—
3	—	.098	1.477	1.575
4	50	.077	.526	.603
5	59	.074	—	—
6	—	.077	.371	.448
7	92	.081	.145	.226
8	50	.099	—	—
9	51	.580	.229	.809
10	88	.089	.846	.935
11	189	.102	.878	.980
12	78	.051	.052	.103
13	54	.135	.425	.560
14	55	.073	.110	.183

better than that of the free or conjugated phenols. Roen,[10] using the Millon reagent, reported an elevation of blood phenols in all cases of uremia regardless of the type of renal failure. Although the height of the blood phenols was generally related to the uremic syndrome, it was not in direct proportion to the uremic state.

A review of the literature concerning the methods for determining blood and urine phenols by Deichmann and Schafer[11] and Volterra[12] showed that there were very few, if any, methods available that were specific, or nearly so, for determination of the phenols in blood and urine. The methods used by Dickes[8] and Roen[10] and earlier by Becher *et al.*,[13] because of the technic used or the non-specificity of the colorimetric reagent, gave phenol values which were high for human blood, if compared with values obtained by Deichmann and Schafer[11] or with those obtained by the method of Schmidt[14] which eliminates most non-phenolic substances that would affect the final colorimetric reading.

The method of Schmidt[14] for determining blood phenols was critically evaluated by the author. Phenol recovery experiments were run on both beef and human blood. Average recovery of free phenol from beef blood was 77% and from human blood, 68%. Determinations were made on 14 normal human blood samples to obtain a point of reference in evaluating data obtained from bloods of uremic patients. Free and conjugated phenols in blood samples from patients diagnosed as having or developing the uremic syndrome from renal disfunction were determined. The results for free, conjugated and total phenols and those for non-protein nitrogen are shown in Table I.

Each clinical case, except No. 9, shows the free phenol value to be within a normal range (.051 to .088 mg %) as determined on the above mentioned series of normal adult blood. (Schmidt[14] presented "normal" free phenol values for pooled human blood ranging between .011 and .041 mg %). Case No. 9 died 8 hours after the blood sample was taken for analysis. At this late stage of the syndrome the conjugating mechanism was undoubtedly failing rapidly, possibly accounting for the increased free phenol value.† It is important to note that, according to the free phenol values, not at any time is there present enough free phenol in the blood to produce the symptomatic picture of phenol poisoning or the uremic syndrome. This is contrary to the evidence of Becher[1] and Becher and Koch.[3] The present author, with Becher and Litzner,[4] Becher *et al.*,[13] Dickes,[8] Roen[10] and Nesbit *et al.*,[15] finds that the conjugated and hence the total phenols increase in the uremic syndrome, although the values given in this report are much lower than those of these authors.

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[10] Roen, P. R., *J. Urol.*, 1944, **51**, 110.

[11] Deichmann, W. B., and Schafer, L. J., *Am. J. Clin. Path.*, 1942, **12**, 129.

[12] Volterra, M., *Am. J. Clin. Path.*, 1942, **12**, 525.

[13] Becher, E., Litzner, S., and Taglich, W., *Munchen. Med. Wochschr.*, 1925, **76**, 1676.

[14] Schmidt, E. G., *J. Biol. Chem.*, 1943, **150**, 69.

[15] Nesbit, R. M., Burk, L. B., and Olsen, N. S., *Arch. Surg.*, 1946, **53**, 483.

† P.M. findings: Liver: Chronic passive congestion. Kidney: Degenerated and resembling bichloride of mercury poisoning.

Summary. In 14 clinical cases involving uremic syndrome herein reported upon, it was found that:

a) The free phenols had little if any effect in producing or intensifying the uremic syndrome. b) During the uremic syndrome the free, conjugated and total phenols did increase, but not significantly enough to warrant their consideration as an important factor in the uremic syndrome. The methods employed in previous reports give reason to doubt the validity of conclusions drawn concerning the relationship of free, conjugated and total phenols to the uremic syndrome.

16660

Fluorimetric and Biological Determination of Estrogens in the Eggs of the American Lobster (*Homarus americanus*).*†

J. Kenneth Donahue.

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The presence of hormones in the tissues and body humors of a variety of invertebrates, both marine and terrestrial, appears to be well-established.[1] Among these, substances having estrogenic potencies are not uncommon.[2] However, satisfactory procedures for producing extracts free of contamination and for testing the potency of such extracts by fluorimetric, as well as the usual biological technics, have only recently become available. This study was made to determine the estrogenic potencies of the eggs of a representative marine invertebrate—the American lobster.

Materials and Methods. A total of 395 g (wet weight), about a quarter of a million eggs, were used, the total yield of 9 specimens. All the eggs were recently spawned and attached to the caudal appendage. The eggs of the lobster remain within the body cavity for nearly a year following ovulation so that the eggs used in this study were all about 11 months old. The extraction procedure follows: The eggs are thoroughly macerated in a Waring Blendor in twice their volume of 95% ethyl alcohol and allowed to stand for 24 hours. The alcohol is filtered off and the residue re-extracted with alcohol for 24 hours. The alcoholic extracts are combined and shaken thoroughly for a half hour with an equal volume of petroleum ether. The ether fraction is removed and the alcoholic fraction is again shaken with ether. Following this, the alcoholic fractions are combined and evaporated to dryness and the deep orange residue is taken up in toluene as a supersaturated solution. For purposes of further purification preceding fluorimetric and biological analysis 5 cc samples were taken of the toluene concentrate. Five cc of the toluene solution are shaken with 10 cc 1N NaOH

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* The cost of this investigation was defrayed by a grant from the Carnegie Foundation Fund for the Advancement of Teaching.

† The author is indebted to Dr. Robert W. Bates, Endocrine Products Division, E. R. Squibb & Sons, for his guidance in working out the chemical procedure and for his invaluable aid in the fluorimetric studies. The laboratory assistance of E. J. Collins is gratefully acknowledged.

[1] Hanström, B., *Hormones in Invertebrates.* Oxford Press, 1939, Chap. 2, p. 22.

[2] Donahue, J. K., *Endocrinology*, 1940, **27**, 149.

and slowly centrifuged for 10 minutes. The oily top layer is carefully removed and kept. The NaOH layer is neutralized to pH 3 with concentrated HCl under a small quantity of toluene. The toluene fraction is removed and kept. The oily fraction from the first NaOH separation is treated 5 times in the manner just described and the toluene fractions combined and reduced in volume to about 5 cc. Where the toluene samples appear slightly cloudy owing to the presence of small amounts of oil, another extraction with NaOH may be required.

Fluorimetric Studies. Colorimetric methods for the quantitative determination of estrogens have been greatly improved[3] but where relatively minute amounts of estrogen are involved, fluorimetric analysis appears to be more sensitive.[4-6] For purely qualitative analysis 0.2 to 0.5 cc of the purified toluene extract were placed in a fluorimetric tube and heated with 1 cc 90% sulfuric acid in a water bath at 80°C for 10 minutes. When held up to a narrow beam of light and viewed at an angle of 90° extracts prepared as described showed a blue-green fluorescence easily discernible with the naked eye.

For quantitative work the procedure outlined by Bates and Cohen was followed.[5] Using a Coleman photofluorometer fitted with a B_1 filter at the photocell, subtracting the non-specific fluorescence developed by a blank solution, and comparing the intensity of fluorescence given by the sample with that developed by a standard solution of estrone, it was estimated that each cc of toluene contained between 30 and 50 international units of activity. However, later determinations made in Dr. Bates' laboratory employing a filter combination consisting of a 420 mu Baird Interference Filter at the light source and a combination of a 520 mu Baird Interference Filter and a 3387 Corning Filter at the photocell gave readings of the order of 5 units per cc of toluene. It is believed that this filter combination eliminates virtually all non-specific fluorescence. On the basis of these readings it is estimated that the original 395 grams of eggs yielded between 400 and 500 units of recoverable activity.

Biological Studies. The response of the 150 g spayed rat to injections or vaginal applications of corn oil extracts of the toluene solutions correlated quite closely to the activity as judged by fluorimetric analysis. Ten 150 g rats were injected subcutaneously with the equivalent of 2.5 cc of toluene solution in 3 divided doses over a period of 3 days. The activity was transferred to corn oil by extracting the toluene solution with NaOH and then neutralizing the NaOH to pH 3 under a small quantity of ethyl ether which was mixed with corn oil and allowed to evaporate at room temperature. Ten more spayed females were given direct applications of corn oil solutions to the vagina in the same dosage as above. In taking smears and making vaginal applications great care was taken to avoid trauma. Within the 3-day period all animals showed a clearing of leucocytes and the appearance of large numbers of nucleated epithelial cells. In the injected series 2 animals showed some cornified cells at the end of 3 days. In the intravaginal group 5 of the 10 animals showed large numbers of cornified cells although in no cases were there full estrus responses. Each animal received the equivalent of 1.2 μg of estrone so that, in view of the observation that the rat is some 10 times less sensitive to estrone than the mouse, the dosage given would be close to the threshold necessary for a full response. Until larger quantities of eggs are obtained and highly concentrated extracts prepared from them the consistent production of full estrus smears must wait. In all animals tested, histological study of the reproductive tracts showed varying degrees of growth of the vaginal epithelium up to 13 cell layers of new tissue but relatively slight growth of the uterine epithelium.

Summary. This preliminary study, utilizing recently developed fluorimetric technics together with standard biological procedures, indicates that the eggs of the American lobster at the time of attachment to the caudal appendage contain estrogenic activity estimated at a minimum of 100 international units per 100 g of eggs. That a substantially increased yield may be affected by further refinements in extraction methods is now being investigated.

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[3] Cohen, H., and Bates, R. W., *J. Clin. Endocrinology*, 1947, **7**, 701.

[4] Finkelstein, M., Hestrin, S., and Koch, W., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 64.

[5] Bates, R. W., and Cohen, H., *Fed. Proc.*, 1947, **6**, 236.

[6] Jailer, J. W., *Endocrinology*, 1947, **41**, 198.

16661

Microdetermination of Steroid Estrogens in Urine by Fluorometry.*

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We have previously described[1] a fluorometric method for determination of estradiol, estrone and estriol in pure solution, which was much more sensitive and accurate than the colorimetric methods used in determining these substances. The fluorometric method was only slightly less sensitive for estradiol and estrone than the biological tests, while estriol could be determined at levels too low for biological assay. In all cases the accuracy of the fluorometric method exceeded that of the biological method and the range of error was found to be ± 3%.

The specificity of the new reaction was high, only very few substances interfering with the determination of the estrogens; and since none of these were phenols they could easily be separated from the estrogens and therefore represented no problem in the assay.

The present paper deals with adaptation of the fluorometric method to determination of estradiol, estrone and estriol in urine.

Experimental. (With the assistance of Mrs. B. Wolman.) The following apparatus and reagents were employed in the tests:

1. Coleman Electronic Photofluorometer, Model 12A, with filters B_2, B_1 (optical), PC_2 and PC_9.
2. All-glass distillation apparatus.
3. Test tubes with ground glass stoppers (Pyrex, 15 ccm).
4. Reagents.
   a. Ether, peroxide, free, redistilled.
   b. Sodium bicarbonate, anhydrous, pro anal.
   c. Sodium carbonate, anhydrous, pro anal.
   d. Hydrochloric acid, concentrated, pro anal.
   e. Sulfuric acid, pro anal., diluted with distilled water 4:5.
   f. Sodium hydroxide, pro anal.
   g. Ethanol, redistilled.
   h. Benzene, triophene free.
   i. Phosphoric acid, purest, s.g. 1.75.
   j. Sodium chloride, pure.
   k. Crystalline estrogens.†

A. *Determining the standard estrogen curves.* The Coleman fluorometer provided with the B_2 primary filter and the PC_2 secondary filter was calibrated with 7 cc of a sodium fluoresceinate solution (0.05 γ / cc), so that the value 80 was recovered on the galvanometer scale.

Crystalline estradiol, estrone and estriol dissolved in pure ethanol 96% were used in preparing the standard curves. Substantially smaller amounts of estrogen were used in determining the standard curve than in our previous study,[1] as the response in the Coleman fluorometer was much higher than

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* We are deeply indebted to Dr. B. Y. Brent for supplying us with estradiol and estrone, and to Dr. Oliver Kamm for estriol.

[1] Finkelstein, M., Hestrin, S., and Koch, W., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 64.

† We are deeply indebted to Dr. B. Y. Brent for supplying us with estradiol and estrone, and to Dr. Oliver Kamm for estriol.

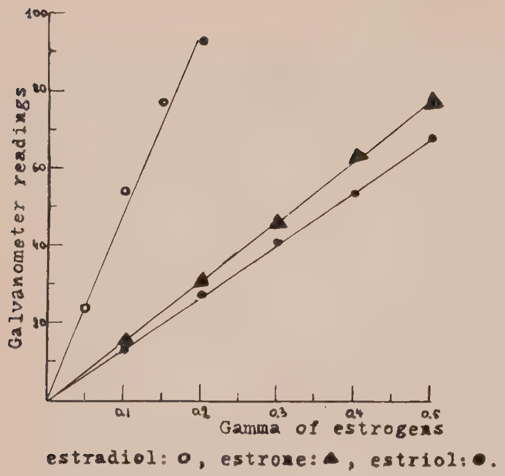

FIG. 1.
The standard curve of estrogens.

that obtained in the Lumetron fluorometer previously employed. The curves of estradiol, estrone and estriol were linear throughout the scale and the limits were 0.05 γ to 0.20 γ for estradiol and 0.1 γ to 0.6 γ for estrone and estriol.

Our procedure in preparing the standard curves was essentially similar to that in our earlier paper, except that 7 cc rather than 2.5 cc of phosphoric acid was used in developing and reading the fluorescence. The standard curves are presented in Fig. 1.

B. *Fractionation of urine in the estradiol-estrone and estriol fraction.* We generally followed the method of Bachmann & Pettit[2] in fractionation though some of the steps in the original method were modified and simplified. Only 10 cc of urine were required in our method, compared with 100 cc in the original method. The recovery percentages, however, fell within the same limits as those described by Bachmann & Pettit.[2] The procedure was as follows:

10 cc of fresh urine were rendered acid with 0.7 cc concentrated HCl (pro anal.) and hydrolyzed under reflux for 1 hour in an all-glass apparatus. The urine was then cooled under running tap water, 5.6 g of sodium chloride was added to it, and it was diluted with 10 cc of distilled water. The solution was extracted 5 times with 20 cc of benzene in a separatory funnel. The pooled benzene extracts (100 cc) were washed in the funnel with 3 cc of sodium bicarbonate 9%, following which the benzene was concentrated to 30 cc. The benzene extract was then shaken in a separatory funnel once with 30 cc and twice with 15 cc of sodium carbonate 9%, and once with 7 cc of water; and the aqueous washings and the sodium carbonate extracts were pooled. Following this procedure all the estradiol and estrone remained in the benzene fraction, while the estriol was almost quantitatively removed in the sodium carbonate fraction. From this point on the two fractions were treated separately.

The estriol fraction. The pooled carbonate extract was rendered acid (less than pH 6) with concentrated HCl and then 3 times extracted with 35 cc of ether. The pooled ether extracts were washed twice in a clean separatory funnel with 10 cc of sodium bicarbonate 9% and twice with 10 cc of water. The ether was then evaporated and the residue was taken up in a measured volume of ethanol, between 10 and 20 cc, and aliquots of this solution were used for fluorometry. In cases when the ether residue was colored, the following additional purification was employed. The residue was taken up in 0.5 cc of ethanol and the solution was diluted with 50 cc of benzene, washed with 2 cc of sodium bicarbonate 9%, and extracted 3 times with half its volume of 0.01 N NaOH and once with 10 cc of water. The pooled extracts were made acid with concentrated HCl and extracted 3 times with half their volume of ether. The ether was evaporated to dryness and the residue was taken up in a measured volume of ethanol, and aliquots of this solution were used for fluorometry.

The estradiol-estrone fraction. The concentrated benzene extract was washed once with a quarter of its volume of dilute sulfuric acid and twice with 15 cc of water. It was then extracted 4 times with an equal volume of N NaOH. The pooled alkaline extracts were acidified with concentrated HCl (pH less than 5) and were then extracted 3 times with 50 cc of ether. The combined

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[2] Bachmann, C., and Pettit, D. S., *J. Biol. Chem.*, 1941, **138**, 689.

TABLE I.
Recovery of Estradiol, Estrone or Estriol from 10 cc Urine Samples.

Estrogen added	γ of estrogen added	γ of estrogen observed after subtraction of the blank value	% of recovery
Estradiol	1	0.6	60
”	10	8	80
”	100	84	84
Estrone	50	37	74
”	50	35	70
”	100	80	80
Estriol	100	56	56
”	100	42	42
”	50	28	56
”	50	30.5	61
”	25	12.5	50
”	25	17	68

extracts were concentrated to about 50 cc, washed once with 10 cc of dilute sulfuric acid, twice with 20 cc of sodium carbonate 9% and twice with 20 cc of water. The ether extract was evaporated, the residue was taken up in a measured volume of ethanol, and aliquots of this solution were used for fluorometry.

C. *The fluorometric measurement.* Aliquots in Pyrex test tubes with ground glass stoppers were evaporated to dryness in an electric oven at about 120°C with the stoppers removed. After cooling, 7 cc of phosphoric acid were added to each, the tubes were stoppered and heated in a boiling water bath in the dark for half an hour. When the tubes were cooled, the fluorescence was measured in the Coleman fluorometer, using filters B_2 + PC_2. For blank readings the measurement was repeated using filter B_1 (optical) + PC_2, and this value was subtracted from the first readings.

Results. In the recovery experiments the tested estrogen was added to 10 cc of hydrolyzed urine. A blank experiment for this sample was run at the same time, and the value thus obtained was subtracted from the experimental reading. Table I summarizes the results of the recovery experiments.

As may be seen from the table, the best estrogen recoveries (above 80%) were obtained with estradiol. As little as 1 γ of estradiol added to 10 cc of urine could be demonstrated with 60% recovery. The recoveries of estrone were in the range of 70%, and those of estriol were lower, between 50 and 60%. Since an inhibitory effect was observed when concentrated aliquots of the estriol fraction were used, we recommend the use of diluted samples of this estrogen for fluorometric assay.

Discussion. The present study describes an accurate and time-saving method for the determination of urinary estrogens, which is also specific and is not influenced by substances accidentally occurring in the urine. Its particular advantage is its sensitivity, which permits the use of a urine sample as small as 10 cc of normal urine and even less of pregnancy urine. The fluorometric readings are not complicated by interfering substances in the urine, as are the colorimetric readings according to Kober.[3] Thus the estrogen concentration may be calculated in a single reading. Recently, Jailer[4] adapted our fluorometric method to the analysis of estrogens in urine. In a preliminary study he reported the recovery of estrone and estradiol, using diluted sulfuric acid instead of phosphoric acid. This modification, however, proved to be of less value than the original method, since estriol was not detectable by this method, and since his recoveries of estradiol and estrone were in the range of 50%. Furthermore, some of the sterolic substances which did not fluoresce when phosphoric acid was used yielded fluorescent products with sulfuric acid.

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[3] Kober, S., *Biochem. J.*, 1938, **32**, 357.

[4] Jailer, W., *Endocrinology*, 1947, **40**, 198.

A detailed study of metabolism of estrogens in normal, pregnant and pathological conditions will be published elsewhere.

Summary. 1. A quantitative method for the determination of estradiol, estrone and estriol in urine, based on the fluorescence of these substances when treated with phosphoric acid, is described. 2. The method is more accurate, sensitive and specific than other known biological or colorimetric tests. 3. The recoveries of estradiol were above 80%; of estrone between 70 and 80%; and of estriol between 50 and 60%. The mean experimental error was ± 10%. 4. The described method permits the determination of estriol in urine in quantities not hitherto detectable.

The author wishes to express his indebtedness to Prof. Bernhard Zondek for his kind interest and support of this investigation.

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Isotopic Studies of Fixation by Rhizobia in Presence of Hemoprotein.*

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Claims of fixation by free-living species of *Rhizobium* in the presence of plant extracts, growth factors and other organic supplements frequently have been made but not verified.[1] The most recent, that hemoprotein from the root nodule can induce fixation *in vitro,* was suggested by the observation that the pigment occurs in the red state during active fixation but disappears from ineffective or old nodules. Although attempts to substantiate the positive experiments failed,[2,3] such negative findings are not necessarily critical. Fixation by pure cultures might be stopped by the accumulation of an intermediate, and pure cultures, long cultivated on combined nitrogen in the laboratory, might lose their ability to assimilate molecular nitrogen. These objections can be overcome by testing for fixation with the stable isotope, N^{15}, and by using species of *Rhizobium* taken directly from the nodules. Whereas the conventional Kjeldahl method requires fixation of 1.0 mg or more for positive claims, the isotopic method readily detects fixation of as little as 0.01 mg and practically eliminates sampling errors.

Materials and Methods. By "pigment" is meant an aqueous extract from nodules actively fixing N_2 and red with hemoprotein. This extract, after filtration through cheesecloth and centrifugation in a Beams centrifuge, is sterilized by passage under pressure through a Seitz filter. The preparations are usually gummy and difficult to filter. In initial experiments with such filtrates fixation was obtained. In agreement with our earlier results[2] the increases in nitrogen content were too small to have been detected by the Kjeldahl method, but were definite with the isotopic technic. Microscopic and cultural tests revealed a large number of organisms, primarily the root nodule bacteria. Inability to secure sterile filtrates did not arise from faulty technic or poor filters since no difficulty was experienced with laboratory cultures of the rhizobia. These observations suggest a sub-microscopic filterable form in the nodule (Almon and Baldwin).[4] We believe, however, it is more likely that the effect

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* Supported in part by grants from the Rockefeller Foundation and from the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

[1] Wilson, P. W., *The Biochemistry of Symbiotic Nitrogen Fixation,* University of Wisconsin Press, 1940.

[2] Niss, H. F., and Wilson, P. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 233.

[3] Virtanen, A. I., Jorma, J., Linkola, H., and Linnasalma, A., *Acta Chem. Scand.,* 1947, **1**, 90.

TABLE I.
Summary of Isotopic Nitrogen Fixation Experiments.

Exp.	Source of pigment	Cultures	Atom % excess N^{15}		
			Initial gas	Inoculated	Uninoculated
	Sterile				
1	Soybean, cowpea	534	8.85	.010	—.001
2	Cowpea	317, 618	10.20	.011	
		Azotobacter		3.5	
		Clostridium*		.36	
		Radiobacter		.009	
		Mixture†		.90	
3	Soybean	317 and			
		Radiobacter	10.80	.010	
4	,,	317, 610, 534		—.016	
		and Radiobacter	10.60	—.016	
5	,,	Clostridium*	8.73	.013	
				.011	
	Nonsterile				
6	Soybean	534	7.88	.045	.062
				.151	.053
7	,,	317, 534	2.84	.040	.048
				—.009	
				—.001	
				—.020	
8	Cowpea	317	8.15	—.001	—.001
				—.002	—.004
9	,,	610	9.40	.012	.014
10	Soybean		10.06		.076
					.076

Increase of 0.050 atom % excess necessary for significant gain.
* Isolated from a filtered but nonsterile nodular extract.
† Mixture of all cultures used in experiment.

of the gum on the physical properties of the filter determines whether a given organism is retained since suitable dilution of the gummy preparation resulted in a sterile filtrate.

This curious filtering property of the root nodule bacteria was unexpected but useful as it provided a convenient method for securing a culture of the organism directly from the nodules for test of its ability to fix nitrogen. The uncertainty of whether other contaminating forms likewise accompanied these "filterable" rhizobia necessitated suitable bacteriological observations for control of the population in such nonsterile cultures. Microscopic stains were made at every stage of the preparation of the pigment to note the decrease in numbers of organisms, and cultural tests were made on the contents of the isotopic flasks at the end of the experiment. *Azotobacter* was tested for by inoculation of Burk's nitrogen-free medium, *Clostridium*, by inoculation of Winogradsky's nitrogen-free medium and incubating in an atmosphere of molecular nitrogen; and *Rhizobium* by plating on yeast water-mineral salts—sucrose agar. All media were incubated for at least two weeks before discarding; if any growth occurred, a microscopic examination was made; if fixation, the culture was further identified.

The species of *Rhizobium* used were: pea culture 317, soybean culture 534, cowpea cultures 610 and 618. Both positive and negative controls were obtained by including *Azotobacter vinelandii*, a species of *Clostridium* isolated from a soybean nodule, and *Alcaligenes radiobacter*. Details of the specific isotopic technics are given by Burris, Eppling, Wahlin, and Wilson.[5] Incubation in the presence of the N^{15} was for 48 hours at 24-29°C.

Results and Discussion. Results from typical experiments are summarized in Table I. The outstanding conclusion is that none

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[4] Almon, L., and Baldwin, I. L., *J. Bact.*, 1933, **26**, 229.

[5] Burris, R. H., Eppling, F. J., Wahlin, H. B., and Wilson, P. W., *J. Biol. Chem.*, 1943, **148**, 349.

of the sterile preparations brought about fixation by the rhizobia or by mixtures of rhizobia and radiobacter, a frequent contaminant of nodules. The positive results with *Azotobacter* and *Clostridium* demonstrate that fixation could occur under the experimental conditions. As shown in *Experiment* 5 fixation is not always obtained with *Clostridium* probably because of the presence of free oxygen and soluble organic nitrogen. Neither of these would prevent fixation by *Azotobacter*. To reduce the level of soluble available nitrogen, one experiment was made using a purified hemoprotein solution and also hemoglobin from blood. Neither induced fixation by the rhizobia, nor did the addition of oxalacetate alter this result.

Nonsterile prepartions did not consistently induce fixation, the positive results being concentrated in two experiments. The results with the nonsterile filtrates are significant for the argument that lack of fixation by pure cultures of the rhizobia arises through loss of this physiological function after laboratory cultivation. In these experiments preparations were obtained that, although filtered, contained numerous rhizobia. These were identified by microscopic examination, cultural characteristics, physiological tests in litmus milk and veal infusion broth, and by inoculation of the host plant. Such organisms, tested for fixation immediately after recovery from the nodule and prior to any transfer on artificial medium, were unable to fix even the minute quantity of nitrogen that can be detected by the isotopic method (Experiment 7, 8, and 9). In the positive trials with nonsterile filtrates (Experiment 6 and 10) species of *Clostridium* demonstrated to be capable of fixing nitrogen were isolated.

Summary. When tested by the sensitive isotopic method, neither an aqueous extract of nodules nor a purified preparation of hemoprotein could induce fixation of atmospheric nitrogen by free-living *Rhizobium*.

Cultures of the root nodule bacteria taken directly from the nodules and tested prior to cultivation on laboratory media did not fix nitrogen even though hemoprotein was present.

In a few experiments, fixation of molecular nitrogen was obtained, but the nodular preparations were shown to contain nitrogen-fixing clostridia.

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Effect *in vitro* of Specific Lipid Fractions of Animal Sera on Psittacosis Virus.

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Casals and Olitsky[1] have reported that a number of neurotropic viruses were inactivated when mixed with various animal sera and incubated at 37°C. Furthermore when these sera were successively extracted with acetone, ether, and hot alcohol-ether, each extract was effective in inactivating virus. This report attempts to confirm these results—using psittacosis virus—and to isolate that lipid fraction having the virus-inactivating property.

Method and materials. Virus: Psittacosis virus originally isolated by Dr. Dorland Davis from a fatal disease in a parrot ("4" strain) was propagated in the allantoic sac of 8-day-old chick embryos. Allantoic fluid was

TABLE I.
Fifty Percent End-point of Psittacosis Virus Incubated at 37° in Presence of Serum, Saline and Sterile Water.

	1 hr	2 hr	4 hr	24 hr
Serum	$10^{-6.0}$	$10^{-6.0}$	$10^{-5.8}$	$10^{-3.6}$
Water	$10^{-6.0}$	$10^{-6.0}$	$10^{-4.2}$	$10^{-1.1}$
Saline	$10^{-6.1}$	$10^{-5.8}$	$10^{-2.4}$	10^{-0}

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[1] Casals, J., and Olitsky, P. K., *Science*, 1947, **106**, 267.

TABLE II.
Average of 50% End-points a Single Pool of the "4" Strain of Psittacosis Virus Incubated for 1 Hour at 37°C in Presence of Serum and Egg Yolk Extracts and Lecithin.

Flow sheet	Avg of LD_{50} of psittacosis in serum extr.	Avg of LD_{50} of psittacosis in egg yolk extr.	Avg of LD_{50} of egg yolk lecithin*	Avg of LD_{50} of vegetable lecithin†
Serum or egg yolk	$10^{-6.1}$	$10^{-6.0}$		
Bloor's reagent filtrate / ppte—denatured protein	$10^{-6.2}$	$10^{-6.1}$		
petrol ether ppte / filtrate —total lipid	$10^{-4.0}$	$10^{-5.8}$		
acetone filtrate MgCl$_2$ ppte / filtrate-phospholipid free-	$10^{-4.3}$	$10^{-6.1}$		
ether ppte—sphyngomyelin-cerebroside	$10^{-4.6}$			
filtrate / lecithin cephalin	$10^{-1.3}$	$10^{-5.8}$		
CdCl$_2$ ppte / filtrate —cephalin-like	$10^{-1.8}$			
NH$_3$ filtrate—lecithin-like	$10^{-0.8}$			
Control of egg and vegetable lecithin			$10^{-5.9}$	$10^{-6.0}$

* Mr. Theriault of National Institute of Health.
† Pfanstiehl vegetable lecithin.

pooled, cultured for contaminants, titrated in mice and quick frozen in sterile ampules in 2 cc lots. The 6BC and Gleason strains of psittacosis virus obtained from Dr. Orville Golub of Camp Detrick were also used.

Serum extracts. Total lipid extracts by Man's method[2] were made of sera from normal chicken, rabbit, horse, sheep and human bloods. The petroleum ether solution of the serum lipids was then concentrated under negative pressure of 28 inches of mercury at a temperature of 35-40°C in a constant small stream of dry nitrogen. Seven volumes of acetone were then added to this concentrated extract and allowed to stand overnight at 4°C. The precipitate was centrifuged at 1500 r.p.m. in a standard centrifuge for 5 minutes, the supernatant poured off and to it was added 1 drop of a saturated solution of magnesium chloride.[3] This was again centrifuged and the supernatant was saved as the phospholipid-free fraction of the total lipid constituents of serum. The precipitate in acetone was then divided into the ether-soluble and ether-insoluble fractions, the latter being saved as the sphyngomyelin-cerebroside fractions of serum. The lecithin-like fraction was isolated from the ether-soluble fraction using Maltaner's[4] modification of the technic of Levene and Rolf.[5] The cephalin-like fraction was recovered in the ether washing of the cadmium lecithin. Each of these 4 fractions—phospholipid-free, sphyngomyelin-cerebroside, lecithin and cephalin—was concentrated and to each was added an amount of sterile distilled water equal to the amount of original serum. The organic solvent was then bubbled off with nitrogen gas. The extracts were in some cases dried completely with nitrogen gas and then taken up with water with no difference in the final titer. No further steps were taken to identify the fractions.[6] Except for the manifestly-necessary last step, great care was taken to avoid exposure to air, temperatures over 40°C, and water.

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[2] Man, E. B., *J. Biol. Chem.*, 1937, **117**, 183.

[3] Sinclair, R. G., Dolan, M., *J. Biol. Chem.*, 1942, **142**, 659.

[4] Maltaner, F., *JACS*, 1930, **52**, 1718.

[5] Levene, P. A., and Rolf, I., *J. Biol. Chem.*, 1927, **72**, 587.

Titrations. The infected allantoic fluids were made up in 10-fold dilutions in each of the above extracts as well as in controls of distilled water, saline, non-infected allantoic fluids and egg yolk extracts. After incubation at 37°C for times varying from 1-24 hours, 0.03 cc were injected intracranially into 6-week-old mice of the NIH stock strain (10-20 mice for each dilution), and the LD_{50} computed by the method of Reed and Muench.

Results. Since the virus was gradually inactivated in any diluent when incubated at 37°C for longer than 4 hours (Table I), the various fractions were compared on the basis of one-hour results. Under these conditions no virus inactivation could be demonstrated by normal sera. Virus-inactivating properties could be demonstrated, however, in lipid extracts of the same sera. Table II, presenting the averages of 15 extractions, demonstrates that the phospholipid-free and sphyngomyelin-cerebroside fractions have a 10-100 fold inactivating effect, that the lecithin-like and cephalin-like fractions decreased the titer 10,000-100,000 fold, and that the lecithin-like fraction was about 10 times more effective than the cephalin-like fraction. Comparable results were obtained with the 6BC and Gleason strains. Controls of water; saline; egg yolk extracts; commercial lecithin, prepared in concentration equal to that expected in serum extracts by bubbling off the organic solvent in the presence of water; all failed to exert any such inactivating effect.

Discussion. This work fails to demonstrate that animal sera can inactivate psittacosis virus, but tends to confirm findings of others[1] that virus inactivation can occur in isolated lipid fractions peculiar to serum. It should be pointed out that different yields with different titres were frequently obtained from

TABLE III.

Average of 50% End-points of a Single Pool of the "4" Strain of Psittacosis Virus Incubated for 1 Hour at 37° in the Presence of Serum and Egg Yolk Extracts and Lecithin.

	Chicken	Sheep 1	Sheep 2	Human 1	Human 1	Chicken 2	Sheep 3	Sheep 4	Sheep 4	Sheep 5	Sheep 5	Sheep 6	Horse	Pooled	Pooled	Avg	Egg yolk 1	Egg yolk 2
Serum	$10^{-6.0}$	$10^{-5.8}$	$10^{-6.4}$	(1)		(1)	(1)						(1)		(1)	$10^{-6.1}$	$10^{-6.2}$	$10^{-5.8}$
Denatured protein	$10^{-6.4}$	$10^{-6.2}$	$10^{-5.9}$			(2)										$10^{-6.2}$	$10^{-6.3}$	$10^{-5.8}$
Total lipid	$10^{-4.0}$	$10^{-4.0}$	$10^{-4.1}$													$10^{-4.0}$	$10^{-5.9}$	$10^{-5.7}$
Phospholipid free	$10^{-4.0}$	$10^{-3.0}$	$10^{-4.3}$	$10^{-3.5}$	$10^{-4.5}$	$10^{-4.1}$	$10^{-4.5}$	$10^{-5.0}$	$10^{-3.5}$	$10^{-4.6}$	$10^{-5.2}$	$10^{-5,0}$	$10^{-4.8}$	$10^{-4.6}$		$10^{-4.3}$	$10^{-6.2}$	$10^{-6.0}$
Sphyngomyelin cerebroside	$10^{-4.6}$	$10^{-5.4}$	$10^{-3.8}$						(3)							$10^{-4.6}$		
Cephalin lecithin	$10^{-1.5}$	$10^{-3.0}$	$10^{-1.8}$	$10^{-3.8}$	$10^{-2.9}$	10^{-0}	$10^{-1.5}$	10^{-0}	10^{-0}	10^{-0}	10^{-0}	10^{-0}	$10^{-3.0}$			$10^{-1.3}$	$10^{-6.0}$	$10^{-5.6}$
Cephalin													$10^{-2.1}$	$10^{-1.8}$	$10^{-1.5}$	$10^{-1.8}$		
Lecithin													10^{-0}	$10^{-1.0}$	$10^{-1.5}$	$10^{-0.8}$		
Egg yolk lecithin			$10^{-5.9}$															
Vegetable lecithin			$10^{-6.0}$															

(1) Greater than 10^{-4}: 100% mortality at 10^{-4}.
(2) Greater than 10^{-6}: 80% mortality at 10^{-6}.
(3) Greater than 10^{-5}: 90% mortality at 10^{-5}.

equal aliquots of the same sera. This may be due to the difficulties in extracting a pure substance that is not contaminated or decomposed.[6]

Summary. Specific lipid fractions of certain animal sera appear capable of inactivating psittacosis virus *in vitro* though the original sera exerts no such effect. The virus inactivating property appears to be in the lecithin-like and cephalin-like fractions, and peculiar to serum lipids.

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[6] Bull, H. B., Biochemistry of the Lipids, 1937.